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14. ABSTRACT Prostate cancer is one of the most resistant tumors to chemotherapy among all adenocarcinomas, and there is virtually no effective therapeutic regimen available for this cancer. Hormonal treatment is the most effective therapy in advanced cancer, however, almost all the patients who undergo hormonal therapy inevitably develop hormone-resistant tumors. Therefore, developing a better therapeutic agent by targeting a specific gene or pathway with well-defined clinical rationale is needed. We chose a target called Fatty acid synthase (FAS) because we found that FAS is strongly expressed in prostate cancer cells but not in normal cells and that inhibiting the FAS expression causes specific tumor cell death. In this project, we plan to test the hypothesis that an active component of Solidago virgaurea specifically inhibits the FAS activity and induces apoptosis in prostate tumor cells. Our specific aims are (i) to elucidate the molecular mechanism of growth inhibitory effect of S. virgaurea by defining the signal pathway and factors responsible for apoptosis, and (ii) to examine the effect of the active component of Solidago virgaurea on tumorigenesis in an animal model.

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INTRODUCTION

Prostate cancer is one of the most resistant tumors to chemotherapy among all adenocarcinomas, and there is virtually no effective therapeutic regimen available for this cancer. The failure of the current approach to develop an anti-prostate cancer drug suggests that we need essentially a new approach by defining a specific target molecule in this cancer. Traditional screening of anti-cancer drugs has been mostly dependent on growth inhibition assay for cancer cells. However, targeting a specific gene with well-defined clinical rationale will provide a better chance of developing a more effective therapeutic agent.

FAS is expressed at low or undetectable level in most normal human tissues, with the exception of lactating breast and cycling endometrium. In contrast, elevated expression of FAS and abnormally active endogenous fatty acid synthesis are characteristics of many human cancers, and the up-regulation of FAS was related in most cases to poor prognosis (2,3). Although the biological basis for this phenotype alteration in cancer cells is not clearly understood, it represents an experimental strategy for cancer therapy because inhibition of FAS is selectively cytotoxic for tumor cells and causes apoptosis. How the inhibition of FAS leads to cell death is an intriguing question. Considering that almost none of the conventional chemotherapeutic agents are effective for prostate cancer, we turned our attention to natural and herbal products that have been used for cancer treatment in different geographic areas. After screening over 100 different herbal plants for their inhibitory effect on the FAS expression, we found that *S. virgaurea* has strong suppressor activities on the FAS gene. The cytotoxic activity of *S. virgaurea* appears to be mediated by inhibition of FAS, which eventually leads to apoptosis. The most intriguing question is how *S. virgaurea* suppresses the expression of FAS. We hypothesize that the active component of *S. virgaurea* suppresses tumor growth by inducing apoptosis through inhibition of FAS and that this inhibitory effect on FAS is mediated by blocking the upstream signal of FAS gene expression including PI3, MAPK and Akt. In this project, we plan to accomplish two specific aims: (i) define the mechanism of cytotoxic activity of *Solidago virgaurea,* and (ii) to examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in a transgenic animal model of prostate cancer

BODY

Task 1a: We will first purify the active component of *S. virgaurea* through a series of column chromatography.

We attempted to further purify the cytotoxic activity of *S. virgaurea* using various chromatographic media and found that a combination of heat-treatment followed by column chromatography of G100 and methyl-HIC (BioRad) can effectively purify the activity. The crude extract of *S. virgaurea* was heated at 80°C for 5min followed by centrifugation. The supernatant was concentrated by the Amicon concentrator and applied on a G100 column. The active fraction of G100 was then applied onto the HIC column which was sequentially eluted with 2.4, 1.8, 1.2, 0.8, 0.6 and 0.3M $(NH_4)_2SO_4$. When each fraction was dialyzed and assayed, we found that the cytotoxic activity was eluted with 1.2 M $(NH_4)_2SO_4$. After repeating the purification steps with G100-sephadex and the HIC chromatography, the final HIC fraction was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig.2,

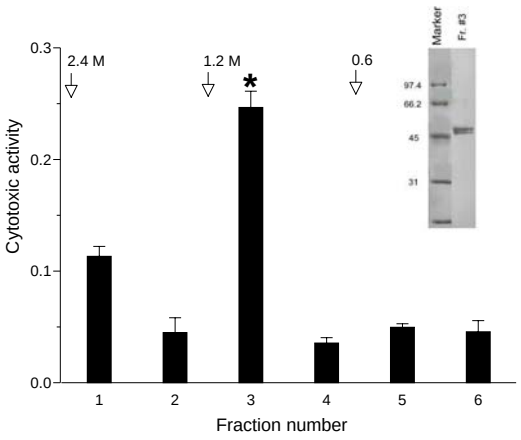


Fig. 1. Purification of cytotoxic activity.
The active fractions of G100 column chromatography were pooled, dialyzed and applied to an HIC column, which was washed and eluted with ammonium sulfate buffer with the indicated salt concentrations. The eluted fractions were assayed for their cytotoxic activities and subjected to SDS-polyacrylamide gel electrophoresis (inset).

the active fraction eluted from the HIC column contained two species of proteins that had close molecular weights around 47-49 kD. We have also tried various traditional column chromatographies including DEAE, HA, phosphate, ConA and heparin agarose. However, these column systems did not retain the active component under all tested conditions. We also tried to purify the protein by using HPLC with C16 column. However, we were not able to recover any activity. We then attempted to purify the two proteins directly from SDS PAGE. The partially purified fraction was run on two lanes of a polyacrylamide gel. After electrophoresis, one lane was cut and proteins were visualized with silver staining. Using the stained lane as a guide, two bands in the unstained gel were excised and gels were extensively washed with buffer to remove SDS and re-nature the proteins. Proteins were then eluted by electrophoresis in a dialysis bag followed by concentration using Centricon P10 which has molecular weight cut-off about 10,000. Each concentrated protein and their combination were then assayed for cytotoxicity. As shown in Table 1 below, we found that the protein with lower molecular weight (48 kD) had significantly more cytotoxic activity, suggesting that the active molecule is a single protein. Based on this information, we are planning to use HPLC again with a sizing column to prepare the pure protein with the 48kD protein.

Table 1. Cytotoxic activity of purified bands

	MW	Cytotoxicity
upper band	49kD	0.086
lower band	48kD	0.210
combination	0.196	

Task 1b: We will examine the status of the FAS signaling pathway upon addition of *S. virgaurea*. We will also examine the expression of various signal molecules using the antibody microarray.

Because the expression of FAS is known to be partly controlled by the Akt pathway, we have examined the effect of *S. virgaurea* on the phosphorylation status of Akt. Human prostate cell line, PC3mm, was cultured in the presence and absence of *S. virgaurea* for 24 hrs. The cells were harvested and the cell lysate was subjected to Western blot using pan- and phospho-specific antibodies (Fig. 2). Our results indicate that *S. virgaurea* indeed strongly inhibited the phosphorylation of Akt as well as the expression of FAS. This inhibition was also accompanied by Caspase 3 activation as shown in Fig.

3. These results indicate that inhibition of FAS expression by S. virgaurea is partly due to the blockade of the Akt pathway and that this blocking induces Caspase 3-dependent apoptosis pathway.

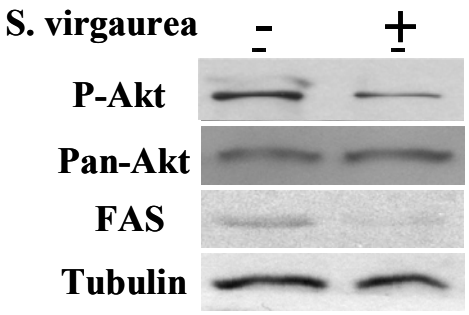


Fig. 2. Inhibition of FAS by S.virgaurea is mediated via Akt pathway. PC3mm cells were treated with or without S. virgaurea for 24 hrs and the cell lysate was subjected to Western blot analysis using antibodies to phospho-Akt, pan-Akt, FAS and tubulin.

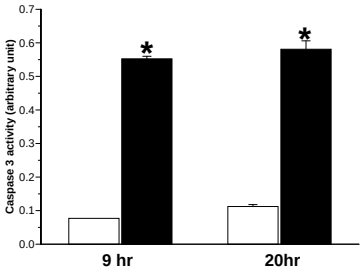


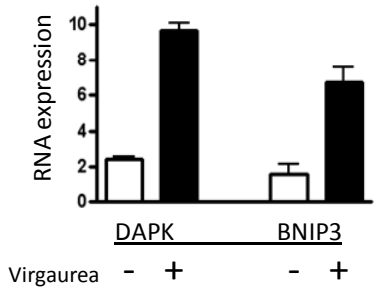
Fig. 3. Caspase assay. PC3 cells (5 x 10^6 cells) were mixed with (closed bar) or without (open bar) the G100 fraction in 1ml of RPMI medium for 9 and 20 hours at 37°C. The cells were then harvested and the cell lysates were tested for Caspase-3 activity by ApoAlert kit (Clontech). Values are mean +/- SD of triplicate

Table 2 Signal array analysis

Gene	fold-suppression
MKK2	2.10
PKB/Akt	1.92
P35	1.91
e-NOS	1.77
MDM2	1.66
Calnexin	1.60
Phosphothronine	1.58
HDAC2	1.55

We also performed an antibody array analysis for tumor cells with or without treatment of S. virgaurea using Panorama Ab microarray system (Sigma Aldrich Co) which contains antibodies to various signaling molecules. The cells were lysed and proteins were labeled with Cy3 or Cy5 followed by hybridization to the antibody array slides. Table 2 summarizes the list of signaling molecules that are significantly suppressed in the tumor cells, PC3mm, that were treated with S. virgaurea. It should be noted that Akt was also identified as a suppressed protein by this analysis. We are currently verifying the result of microarray by Western blot. Because this array analysis has limited number of antibodies, we also plan to use Kinxus signaling array analysis.

Task 1c. **We will examine the status of Malonyl-CoA, ceramide and expression of the pro-apoptotic genes, BNIP3, DAPK2 and TRAIL as well in response to *S. virgaurea*.**



We have previously shown that inhibition of FAS expression by shRNA accumulated ceramide and induced BNIP3, DAPK2 and TRAIL. We expected that *S. virgaurea* shows a similar effect on prostate tumor. To test this possibility, we have tested the effect of S. virgaurea on the expression of BNIP3 and DAPK2 by qRT-PCR. As shown in Fig. 4, we found that S. virgaurea indeed up-regulated the expression of both BNIP3 and DAPK2, suggesting that apoptosis induced by S. virgaurea is mediated by up-regulation of these pro-apoptotic genes.

Task 2. To examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in a transgenic animal model of prostate cancer

We have been waiting for the purified compound of Solidago virgaurea before pursuing Task 2. Due to the delay of Task 1, we are somewhat behind schedule. However, we expect to catch up with the pace next year.

While we were waiting for the progress on Task 1, we also pursued a possibility of using another natural product, *Cacalia deliphiniifolia*, for prostate cancer therapy. We have identified anti-FAS activity of *Cacalia deliphiniifolia* when we initially screened various natural products for the current project. The results of the screening identified Solidago virgaurea which showed the highest anti-FAS activity as we described in the current project. However, the extracts of *Cacalia deliphiniifolia* also showed strong anti-FAS activity in our in vitro assay. Therefore, to accomplish the overall goal of our project which is to identify natural compounds to block FAS activity, we also decided to study *Cacalia deliphiniifolia* in parallel. As shown in Fig. 4a. extracts of *Cacalia deliphiniifolia* significantly inhibited the expression of FAS in a prostate cancer cell, PC3mm, in a target specific manner. This inhibition of FAS expression also induced apoptosis measured by TUNEL assay (Fig. 4b).

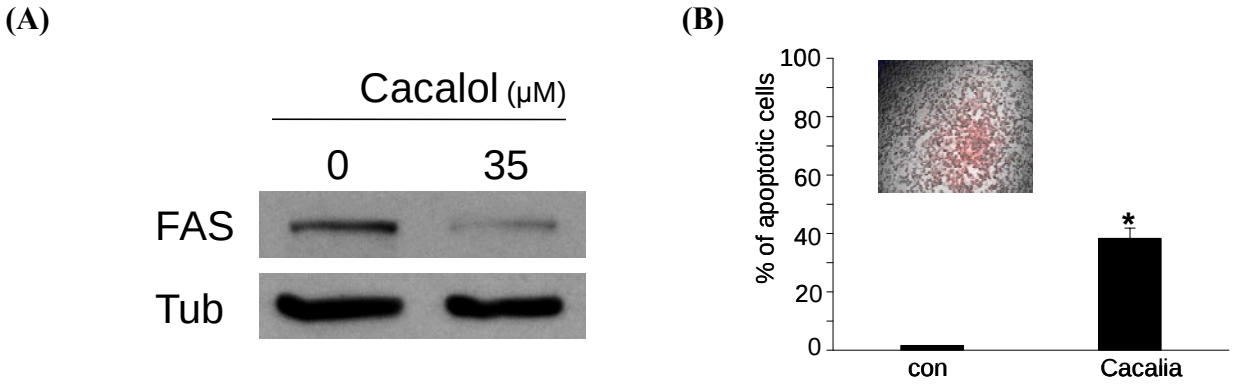


Fig. 4. *Cacalia deliphiniifolia* inhibits FAS expression and induces apoptosis. PC3mm cells were cultured in the presence or absence of *Cacalia* for 24 hrs. Cell extracts were then subjected to Western blot analysis using FAS-specific antibody (A). The cells were cultured in 96-well plate and treated with or without *Cacalia* for 36 hrs. They were then assayed for apoptosis using Cell death TMR kit (B).

We then purified the active component through series of TLC and HPLC and found that the active compound is identical with cacalol which was previously found to have strong anti-oxidant activity. We are currently testing an anti-tumor effect of this compound in animal using both prostate and breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

1. We have found that *Solidago virgaurea* blocked phosphorylation of Akt followed by inhibition of FAS expression.
2. This inhibition of Akt up-regulated the expression of BNIP3 and DAPK followed by activation of Caspase 3 and induction of apoptosis.
3. The bioactive protein of *Solidago virgaurea* was purified and found to be a single peptide with a molecular weight of 48kD.

4. We found another natural product, *Cacalia deliphiniifolia*, which blocks FAS expression and induces apoptosis. The active compound was purified and found to be identical with cacalol. We will also pursue this product as a part of this project in the following years.

REPORTABLE OUTCOMES

Peer reviewed publications

(The following works were directly or partly supported by the current grant)

1. Furuta, E., Pai, SK., Zhan, R., Bandyopadhyay, S., Watabe, M., Iiizumi, M., Liu, W., Mo, Y-Y., Hirota, S., Hosobe, S.,Tsukada,T., Miura,K., Kamada, S., Saito, K. and Watabe, K. (2008) Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and SREBP. *Cancer Res.* 68, 1003
2. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K Pai, Misako Watabe, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Wen Liu, and Kounosuke Watabe (2008). RhoC promotes metastasis via activation of Pyk2 pathway in prostate cancer. *Cancer Res.* 68(18):7613-20.
3. Megumi Iiizumi, Wen Liu, and Kounosuke Watabe. (2008) Drug development against metastasis-related gene and their pathways: A rationale for cancer therapy. *Biochim. Biophys. Acta. Cancer Review 1786*, 87-104.

Abstract/presentation

1. Eiji Furuta, Rui Zhan, Sucharita Bandyopadhyay, Shigeru Hirota, Sadahiro Hosobe, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Sonia Mohinta, Wen Liu, Kounosuke Watabe. Hypoxia induced ROS up-regulates the fatty acid synthase gene via Akt pathway in breast cancer cells. (2008) Annual meeting of American Association for Cancer Research. San Diego, CA
2. Wen Liu, Eiji Furuta, Misako Watabe, Kazutoshi Shindo, Megumi Iiizumi, Sudha Pai, Kounosuke Watabe. (2008) Inhibition of Fatty acid synthase and induction of apoptosis in human breast cancer cells by Cacalia deliphiniifolia Annual meeting of American Association for Cancer Research. San Diego, CA
3. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K Pai, Misako Watabe,Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Wen Liu, and Kounosuke Watabe (2008) RhoC promotes metastasis but not growth of prostate tumor. Annual meeting of American Association for Cancer Research. San Diego, CA
4. Wen Liu, Sucharita Bandyopadhyay, Eiji Furuta and Kounosuke Watabe (2008) Role of tumor metastasis suppressor gene, NDRG1, in breast cancer progression. DOD Breast Cancer Research Program, Era of Hope 2008 Meeting. Baltimore MA
5. Fei Xing, Eiji Furuta, Misako Watabe, Sudha K Pai, Wen Liu, Puspa Pandey, Hiroshi Okuda, Aya Kobayashi, Megumi Iiizumi and Kounosuke Watabe Notch pathway is stimulated by hypoxia and promotes metastasis through activation of EMT and metalloproteinase. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
6. Hiroshi Okuda, Eiji Furuta, Misako Watabe, Sudha K. Pai, Wen Liu, Aya Kobayashi, Fei Xing, Puspa Pandey, Megumi Iiizumi and Kounosuke Watabe The expression of metastasis suppressor gene, KAI1/CD82, is down-regulated by OCT4, SOX2 and NANOG in tumor stem cells of breast cancer. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
7. Wen Liu[1], Eiji Furuta[1], Misako Watabe[1], Kazutoshi Shindo[2], Fei Xing[1], Sudha Pai[1], Hiroshi Okuda[1], Megumi Iiizumi[1], Puspa Pandey[1], Aya Kobayashi[1], Kounosuke Watabe[1]Inhibition of fatty

acid synthase and induction of apoptosis in human breast cancer cells by Cacalia deliphiniifolia. 2009 Annual meeting of American Association for Cancer Research. Denver Co.

8. Eiji Furuta, Puspa R. Pandey, Hiroshi Okuda, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Wen Liu, Fei Xing, Aya Kobayashi, Kounosuke Watabe Resveratrol induces apoptosis by blocking enzymatic activity and destabilizing the protein of fatty acid synthase in breast tumor cells. 2009 Annual meeting of American Association for Cancer Research. Denver Co.

Employment

1. Ms. Wen Liu (Graduate student) has been supported by the current grant.
2. Dr Eiji Furuta (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

During the last funding period, we focused our major effort to purify the active compound. After many trials and errors, we were able to identify the active protein which has 49kD. Therefore, we consider that Task1a is accomplished, although we still need to obtain a large amount of this protein for the animal experiment as proposed in Task2. We also found that S virgaurea blocks the Akt pathway which suppresses FAS expression followed by inducing apoptosis by activating pro-apoptotic genes, BMP3 and DAPK. Therefore, we consider that Task 1b is accomplished and Task 1c is also almost done although it is still incomplete. We need large scale purification of the active protein from S. virgaurea to execute Task 2 which is currently underway. Meantime, we found that another natural product, *Cacalia deliphiniifolia* also showed strong anti-FAS activity in prostate cancer cells. We identified the active compound as cacalol which has strong anti-oxidant activity. This is particularly interesting because we can expect synergistic effect of *Solidago virgaurea* and *Cacalia deliphiniifolia* on FAS expression which may have significant impact on prevention of prostate cancer by proper diet.

So what?

Our preliminary data indicate that FAS is considered to be an ideal target for this purpose. *S. virgaurea* has been used as herbal medicine in the past to treat urological diseases and known to be non-toxic. Our discovery of specific inhibition of FAS activity by the extract of *S. virgaurea* suggests a potential utility of this traditional medicine as a chemopreventive as well as therapeutic remedy for prostatic cancer. During this funding cycle, we successfully isolated the active protein from S. virgaurea. We also identified a possible signaling pathway by which S. virgaurea induces apoptosis of prostate cancer cells. We believe that these findings provide strong rationale to use S. virgaurea as anti-cancer therapy. Furthermore, our new finding that cacalol from *Cacalia deliphiniifolia* showed a similar effect on FAS and induced apoptosis in prostate tumor cells adds another layer of interest to this project because of the potential utility of both products as non-toxic chemopreventive agents for prostate cancer.

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Mol Cancer Res.1:707-15.

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Review

Drug development against metastasis-related genes and their pathways: A rationale for cancer therapy

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ABSTRACT

It is well recognized that the majority of cancer related deaths is caused by metastatic diseases. Therefore, there is an urgent need for the development of therapeutic intervention specifically targeted to the metastatic process. In the last decade, significant progress has been made in this research field, and many new concepts have emerged that shed light on the molecular mechanism of metastasis cascade which is often portrayed as a succession of six distinct steps; localized invasion, intravasation, translocation, extravasation, micrometastasis and colonization. Successful metastasis is dependent on the balance and complex interplay of both the metastasis promoters and suppressors in each step. Therefore, the basic strategy of our interventions is aimed at either blocking the promoters or potentiating the suppressors in this disease process. Toward this goal, various kinds of antibodies and small molecules have been designed. These include agents that block the ligand-recepter interaction of metastasis promoters (HGF/c-Met), antagonize the metastasis-promoting enzymes (AMF, uPA and MMP) and inhibit the transcriptional activity of metastasis promoter (β-Catenin). On the other hand, the intriguing roles of metastasis suppressors and their signal pathways have been extensively studied and various attempts have been made to potentiate these factors. Small molecules have been developed to restore the expression or mimic the function of metastasis-suppressor genes such as NM23, E-cadherin, Kiss-1, MKK4 and NDRG1, and some of them are under clinical trials. This review summarizes our current understanding of the molecular pathway of tumor metastasis and discusses strategies and recent development of anti-metastatic drugs.

Contents

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[1] MI and WL contributed equally to this article.

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1. Introduction

Cancer is the second leading cause of death in the USA, and more than half a million people succumb to the disease every year [1]. Despite significant improvements in screening methods and treatment options, the majority of cancer patients are still diagnosed at an advanced stage, and more than 90% of patients ultimately die from sequel of metastatic disease. Therefore, metastasis is a hallmark of malignancy, and no effective therapeutic option is currently available for those patients. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that cancer cells are extremely heterologous in nature and that metastasis involves multiple steps with a high degree of complexity, and each step requires coordinated action of many promoters and suppressors. However, extensive efforts in the past decade have led to the discoveries of many previously unknown factors involved in metastasis and also unveiled several novel concepts in this research field [2,3]. These findings have shed new light on molecular pathways of metastasis, which also provided valuable information about potential targets for the treatment of metastatic disease. This review discusses our current understanding of molecular mechanism of metastatic process and summarizes recent information of drug development specifically targeted to the metastatic pathways.

2. Tumor metastasis involves multi-step process with high complexity

A primary tumor generally consists of heterogeneous cell types including a small number of cancer stem cells that are able to perpetually proliferate without responding to tumor suppressor function. The current theory predicts that these cancer stem cells originate from a normal stem cell or a cancer cell, which acquired a stem cell-like ability [4]. When a tumor grows more than 1 mm^3 in size at the primary site, it acquires active supply of oxygen and nutrients by promoting angiogenesis. Tumor cells accomplish this task by generating hypoxic environment followed by secretion of angiogenic growth factors (Fig. 1). Tumor cells that gain growth advantage further proliferate and acquire metastatic phenotypes due to additional mutations. The first step in metastasis is the detachment of these tumor cells from the primary tumor mass by acquiring an invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix (Fig. 1) [5]. It is believed that autocrine motility factor (AMF) and hepatocyte growth factor (HGF) are critical components of motility and that degradative enzymes including serine-, thiol-proteinases, heparanases and metalloproteinases such as MMP2 and 9 play critical roles in the invasion [6–8]. When tumor cells intravasate surrounding tumor vasculature and neighboring lymphatic vessels, they must survive in this hostile environment that includes mechanical damage, lack of growth factor from the original environment and the host immune system (Fig. 1) [9]. Tumor cells in the circulation often aggregate with platelets and fibrin, and they embolize in the capillaries or directly adhere to the endothelial cells by a mechanism similar to leukocyte adhesion at the inflammatory site [10–12]. In some cases, arrested tumor cells extravasate before proliferating themselves using the same hydrolytic enzymes that are used in the initial step of invasion (Fig. 1) [13]. However, in many cases, cancer cells actually proliferate within the lumen of vessels to create a considerable tumor mass that can eventually obliterate the adjacent vessel wall by pushing aside the barrier composed of endothelial cells, pericytes and smooth muscle cells that previously separated the vessel lumen from the surrounding tissue [14,15]. After extravasation, cancer cells lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis (Fig. 1). These processes are controlled by various metastasis promoters and suppressors, and they must be well coordinated to establish successful distant metastasis (Table 1) [2]. Recent advancement of research in this field has revealed the complex interplay of metastatic factors and many novel concepts of signal pathways leading to metastasis (Fig. 2 a,b). Based on this information, the current research is gradually moving toward translational stage by aiming at development of targeted anti-metastatic drugs (Table 1). The following sections summarize up-to-date information of the promoters and suppressors of metastasis that are currently under active investigation for drug development.

3. Metastasis promoters

3.1. Amf

Autocrine motility factor (AMF) was originally isolated as a C-*X*-*X*-C cytokine that stimulates random or directed motility of AMF-

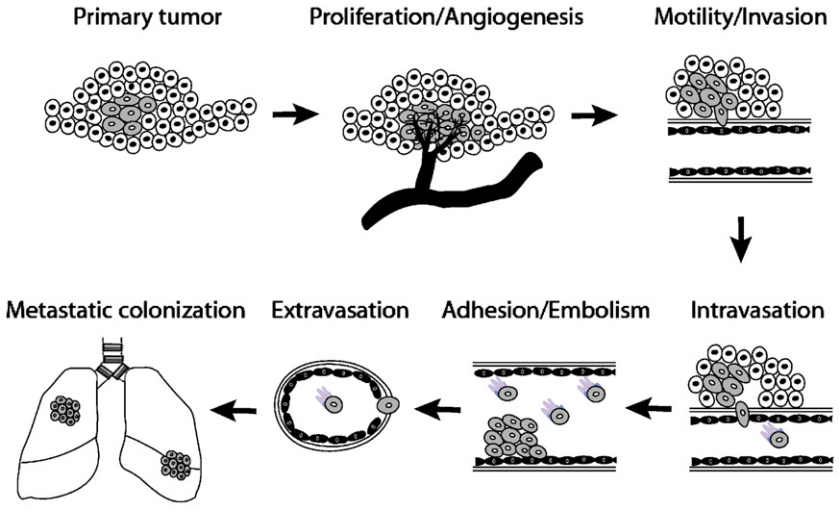

Fig. 1. Process of tumor metastasis. As primary tumor grows, tumor cells induce angiogenic factors to promote vessel formation which facilitates tumor growth and cell invasion into the circulatory system. Some tumor cells gain an invasive ability by expressing motility factors and proteases followed by breaching the basement membrane. Tumor cells then enter the blood vessel where they often aggregate with the platelets and cause embolize. When cells migrate to a distant organ, they adhere to endothelial cells and extravasate by inducing proteases. Cells then colonize and establish metastasis at the distant organ site where appropriate growth factors are provided.

producing tumor cells in an autocrine manner [16]. Elevated serum AMF was found in patients with malignant tumors such as colorectal, lung, kidney, breast and gastrointestinal carcinomas and is well correlated with the development of metastasis [16–19]. AMF is a multifunctional molecule, also known as phosphoglucose isomerase, neuroleukin, and maturation factor [20]. AMF causes tumor cell detachment from the primary site by promoting cell motility in an autocrine fashion. However, recent research revealed that AMF also contributes to malignant progression by stimulating the migration and proliferation of endothelial cells via its receptor AMFR, a unique seven transmembrane receptor (gp78), followed by activation of small Rho-like GTPase [16,21]. Therefore, tumor cells appear to induce aggressive angiogenesis by promoting cross-talk of signals between VEGF-VEGFR and AMF-AMFR which also promotes cell survival via activation of Akt and MAPK-dependent anti-apoptotic pathways (Fig. 2) [22]. A recent report by Raz et al. demonstrated a more direct role of AMF in tumor progression and metastasis. They have shown that over-expression of AMF in normal fibroblasts lead to a gain of tumorigenicity, whereas down-regulation of AMF by siRNA in mesenchymal tumor cells resulted in mesenchymal-to-epithelial transition (MET), the reverse process of epithelial-to-mesenchymal transition, as reflected by a loss of cell polarity, reduced proliferation and invasion *in vitro* and loss of tumorigenic properties *in vivo* [23]. Interestingly, they later also showed that silencing AMF expression in human fibrosarcoma cells resulted in an increased sensitivity to oxidative stress-induced and p21-mediated cellular senescence, which brought a novel insight into the function of AMF in tumor progression [24]. Collectively, neutralizing AMF, disruption of AMFR and blocking their signal pathways are considered to be rational approaches for anti-metastatic drug development.

It has been shown that specific carbohydrate phosphate inhibitors including E4P, D-mannose-6-phosphate and 5-phospho-D-arabinonate (5PAA) are able to block both AMF enzymatic activity and AMF-induced cell motility [25,26]. Treatment of tumor cells with these inhibitors has been shown to decrease the growth, DNA synthesis, migration and invasiveness of several types of cancer cells [22,23,27]. Since these carbohydrate phosphate inhibitors are among the smallest compounds that have AMF inhibitory activity, information of the known crystal structure may help in designing a lead compound to develop more effective AMF inhibitors.

Because AMF is a secretory factor, antibody against AMF may also be a rational approach. In fact, Talukder et al. showed that neutralizing antibodies against AMF were able to partially block HRG-induced invasiveness of human breast cancer MCF-7 cells [28]. Raz et al. also demonstrated that a monoclonal anti-AMF antibody induced apoptosis in human fibrosarcoma cell lines *in vitro* and effectively promoted drug-induced apoptosis *in vivo* [22]. Therefore, humanized anti-AMF holds promise for future therapeutic application. Interestingly, antibody against EGFR2 (Herceptin) was also shown by Talukder et al. to block AMF expression and its promoter activity [27]. Because Herceptin has been used as an effective drug for breast cancer, it is interesting to know whether this antibody also blocks the invasiveness of the tumor.

Ectopic expression of AMF makes some tumor cells become resistant to apoptosis induced by serum deprivation, and this resistance appears to be mediated via PI3K and PKC/MAPK pathways (Fig. 2A). Yanagawa et al. recently indeed showed that PI3K inhibitors (Ly294002 and Wortmanin), PKC inhibitor (GF109203) and MAPK inhibitor (PD98059) were able to recover the expression of Apaf-1 (Apoptotic protease activating factor 1) in the AMF-transfected HT1080 cells followed by induction of apoptosis [22]. In addition, GF109203X and Wortmanin were shown to inhibit AMF-induced expression of fms-like tyrosine kinase (Flt-1) and hence impair the proliferative signals of VEGF in endothelial cells. Therefore, AMF may be a good target for anti-angiogenic therapy, although potential side effects of such drugs are unknown. Finally, it is recently found that the stability of AMF protein is regulated through ubiquitin-lysosome system, which is mediated by poly (ADP-ribose) polymerase-14 (PARP-14). This new discovery may offer a novel target to block the AMF/AMFR signaling and deserves further investigation [29].

3.2. Hgf/sf

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was identified as the natural ligand for the c-Met receptor tyrosine kinase [30]. HGF/SF interacts with c-Met receptor and transduces multiple biological signalings that control proliferation, disruption of intercellular junctions of EMC, migration and protection from apoptosis [31,32]. HGF/SF signaling has also been demonstrated to play an important role in a wide variety of human cancers of both epithelial and mesenchymal origins [31]. The results of several clinical studies indicate the prognostic value of HGF/SF and c-Met in various types of cancer and that the expression of HGF and/or c-Met is frequently associated with the aggressive nature of the tumors and the poor clinical outcome [31,33]. The exact mechanism of up-regulation of these genes in cancer is not well understood. However, a recent study suggested that the up-regulation of c-Met and HGF may be due to the stress of tumor microenvironment such as hypoxia [34]. Therefore, HGF/SF is considered to be widely involved in the tumor metastatic process. HGF is a potential promoter of cell invasion by directly stimulating the motility and migration of cancer cells as well as affecting the microenvironment [32]. HGF can disrupt cell-cell adhesion and promote cancer cell growth, partly by inducing phosphorylation of β-Catenin and relocation of E-cadherin, which may result in down-regulation of cell cycle regulatory factors such as p27 (Fig. 2A) [35-37]. On the other hand, HGF can increase the adhesion between cancer cells and matrix by activating the FAK and paxillin pathways, which cooperatively regulate the expression of integrins in cancer cells and eventually lead to adhesion as well as migration of cancer cells to matrix [38]. HGF is also able to increase the expression and secretion of proteolytic enzymes from cancer cells including MMP2, MMP7, MMP9 and uPA that are involved in matrix and basement membrane degradation (Fig. 2) [36,39,40]. In addition, HGF is considered as an angiogenesis-promoting factor through its direct morphogenic and adhesive effects and indirect regulation of other angiogenic factors such as IL-8, VEGF and TSP-1 [41,42]. Furthermore, Boccaccio et al. have recently demonstrated that the c-Met oncogene was responsible for the induction of thrombohemorrhagic syndrome, suggesting that c-Met may give survival advantage to tumor cells in the circulation by promoting the aggregation of tumor cells with platelets [43,44]. Therefore, the HGF/c-Met signaling plays a critical role in the metastatic process and this gene as well as the downstream signal can be potential targets for cancer therapy.

Recently, rapid progress has been made toward drug development against HGF/SF for the purpose of cancer therapy. These include HGF antagonists, anti-HGF and anti-cMet antibodies, small molecules targeting c-Met and its signaling pathways as well as compounds interfering with HGF-elicited biological activities [45]. Antagonizing ligand binding that block the activation of downstream signaling is a conventional therapeutic strategy for most carcinomas. NK4 is one of the antagonists that compete with HGF for the c-Met receptor, and it has been known to block HGF-induced cellular adhesion, invasion and metastasis in various types of cancer cells including breast, bladder, colorectal, lung, prostate, glioma, pancreatic and gastric cancers *in vitro* [46]. Moreover, NK4 also acts as angiogenesis inhibitor, and this activity is independent of its action as HGF-antagonist [47,48]. As expected, treatment of mice via intraperitoneal or intratumoral administration of NK4 protein or recombinant adenoviruses expression vector effectively blocked tumorigenesis, angiogenesis and metastasis in various mouse xenograft models including pancreatic and gastric cancers [46,49]. Another antagonist is an uncleavable HGF, which was engineered with a single amino-acid substitution at the

Table 1

Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
AMF	carbohydrate phosphate compounds (E4P,M6P,5PA)	AMF	Inhibit AMF cytokine enzymatic activity		Pre-clinical studies	[25,26]
	Herceptin	EGFR2	Down-regulates AMF protein and promoter activity	Increase the tumor progression time in mice model of xenograft tumor of Her2 over-expression	In clinical use	[27,270]
HGF/c-Met	NK4	HGF	competitive antagonist for HGF binding to the c-Met receptor	Inhibited tumorigenesis, angiogenesis and metastases in mouse tumor xenograft models	Pre-clinical	[46,49]
	uncleavable HGF	HGF	Prevent maturation of pro-HGF and compete with HGF to bind to c-Met receptor	Inhibited tumor growth, angiogenesis and metastases in tumor xenograft models	Pre-clinical	[50]
	AMG102	HGF	Neutralizing anti-HGF antibody	pharmacokinetic and safety profile are passed through in cynomolgus monkeys test	Phase II	[53]
	DN30	c-Met	Binds to extracellular domain of c-Met and prevent its activation	inhibited growth and metastatic spread to the lung of tumor xenograft mouse model	Pre-clinical	[61]
	PHA-665752 SU11274 K252a	Kinase inhibitors	inhibit c-Met phosphorylation	Inhibition of tumor growth in c-Met-dependent lung and gastric carcinoma xenograft animal model	Pre-clinical	[55-60]
TGF-β	SD-208	TGFβ1 receptor	TGF-β typeI receptor kinase inhibitor	Inhibited primary tumor growth, angiogenesis and metastasis of xenograft animal model	Pre-clinical studies	[68,73,87-92]
	SD-093 SB-431542 A-83-01 LY2109761					
	2G7	TGFβ	Neutralizing antibody of TGFβ	Inhibited abdominal and lung metastasis of xenograft animal model	Pre-clinical	[69]
	β- glycan (sRIII)	TGFβ	Soluble extracellular domain of TGF-β type III receptor	Inhibited lung metastasis in human breast tumor xenograft model	Pre-clinical	[96]
	Fc:TβRII	TGFβ	Dominant negative TGF-β typeII receptor	Inhibited lung metastasis in human melanoma xenograft model and MMTV-Neu model	Pre-clinical	[94,95]
	AP12009	TGFβ	Oligonucleotide against human TGFβ2		PhaseI/II (high grade glioma)	[97]
MMP	Marimastat (BB-2516)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseII,III,IV (Pancreatic cancer) phaseIII Non-small-cell lung cancer)	[122,271]
	Prinomastat (AG3340)	MMPs	inhibitor with selectivity for MMPs 2, 3, 9, 13, and 14	enhance tumoricidal activity after Photodynamic therapy in a mouse mammary tumor model	Phase III, IV (NSCLC) phaseII (advanced esophageal cancer)	[122,272]
	Tanomastat(BAY12-9566)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII (Small-cell lung and pancreatic cancer)	[122,271]
	BMS-275291Neovastat	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII, IV (Non-small-cell lung and Renal cell carcinoma)	[122,271]
	Bisphosphonates (BP)	for use in disorders of bone metabolism	Inhibit proteolytic activity of MMPs	Increase bone mineral density in animal model	In use (osteolytic metastases)	[129]
uPA	WX-UK1 WX-671	uPA	Protease inhibitor		Phase I,II	[148]
	231 Bi-PAI2	uPA	Recombinant PAI-2 (uPA inhibitor-2)	Inhibited micrometastasis in human breast cancer xenograft models	Pre-clinical studies	[160-162]
	1-Isoquinolinylguanidines (UK-356,202) and its derivatives	uPA	Reversibly competitive inhibitors of uPA enzymatic activity	Inhibit exogenous uPA in human chronic wound fluid and in the porcine excisional wound model	Pre-clinical studies	[273]
	Bikunin	Trypsin and plasmin	Down-regulate uPA gene and protein expression	once-daily oral administration of bikunin against ovarian carcinoma in nude mice	Phase I	[153-156]
	DX-1000 PEGylated DX-100	plasmin	Down-regulate uPA expression	Inhibited tumor proliferation and vascularization in human tumor xenograft model	Pre-clinical	[157,158]
β-catenin	Celecoxib	COX-2	Induce degradation of β-catenin via a COX-2-independent mechanism	Diet treatment significantly reduce tumor development without signs of metastasis in TRAMP mice	phase II (advanced colorectal cancer)	[182,183,274]
	R-Etodolac and its analog (SDX-308)	enantiomer of Etodolac	Down-regulates protein and promoter activity, increase β-catenin and E-cadherin complex at the membrane	inhibited tumor development and metastasis in the transgenic mouse adenocarcinoma of the prostate (TRAMP) model	phase II (chronic lymphocytic leukemia)	[182,183]
	Thiazolidinedione (TZD)	PPARs	cause localization shift to cytoplasm, reduced tyrosine phosphorylation of beta-catenin	Inhibited lymph node and lung metastases in the xenograft animal model	Pre-clinical studies	[185]

Table 1 (*continued*)

Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
β-catenin	Exisulind(Aptosyn)	SAANDs	Down-regulate β-catenin and cyclin D1 via PKG-mediated signalling	Inhibited tumor growth and metastasis of human lung cancer xenograft in athymic nude rats.	Phasae I,II,III	[164,172,173]
	CP461 CP248					
	Imatinib (Gleevec)	PDGF receptor	Inhibits tyrosine phosphorylation of β-catenin and resultant cell migration		In use (chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) etc)	[176]
Metastasis Suppressor NM23	Medroxyprogesterone acetate (MPA)	Progesterone receptor	MPA elevated NM23 expression and inhibited soft agar colonization	Inhibited lung cancer metastasis in the experimentally metastasis mice model	Phase III(metastatic breast cancer)	[206,275,276]
	Estradiol	Estrogen receptor	Up-regulates NM23-H1 in ERa+ breast cancer cell lines. Inhibits invasion *in vitro*.	Suppression of lung metastasis *in vivo* model of chemically induced hepatocellular carcinoma.	Phase II (metastatic breast and prostate cancer)	[212,277]
	Aspirin	Cox1/2 inhibitor	Up-regulates NM23. Decreased metastatic phenotype *in vitro*.		Phase III (esophageal cancer)	[217,277]
	Indomethacin	Cox1/2 inhibitor	Up-regulates NM23 expression in breast cancer cell lines	Inhibited lung tumor metastasis in the experimental metastasis mice model	Phase II (head and neck cancer)	[219,220,277]
	All-trans retinoic acid (ATRA)	Retinoid receptors	Up-regulates NM23 in hepatocarcinoma cells. Increased adhesion to ECM *in vitro*	Inhibits the growth of xenograft tumors and gastric cancer cell metastasis to liver.	Currently in clinical use, (acute promyelocytic leukemia)	[226-228,278]
KiSS-1	Metastin	orphan G-protein coupled receptor	Regulate the NFκB signaling pathway		Pre-clinical studies	[279]
MKK4	Anti-death receptor antibody (2E12, TRA-8)	death receptor	Induce apoptosis *in vitro*. Activate MKK4/JNK/p38 pathways		Pre-clinical studies	[241]
	Bisindolylmaleimide VIII	PKC inhibitor	Enhances affects of anti-death receptor antibodies		Pre-clinical studies	[245]
E-cadherin	pyrazolo [3,4-d] pyrimidines (PP)1, PP2	Src family inhibitor	Reactivate the E-cadherin expression. Reduced migration ability of breast cancer cells	Decrease in pancreatic tumor growth and metastasis in nude mice	Pre-clinical studies	[255,256,280]
NDRG1	Fe chelator (DFO, 311)	Fe	NDRG1 was specifically up-regulated by Fe chelation.	Delay or regression of tumor cell growth in athymic nude mice.	Phase II (Neuroblastoma)	[263,266,281,282]

proteolytic site of HGF [50]. The uncleavable HGF competes with endogenous pro-HGF for the catalytic domain and thus inhibits endogenous pro-HGF maturation. The peptide also binds to the c-Met receptor with high affinity and displaces the mature ligand. More strikingly, both local and systemic administration of uncleavable HGF in a xenograft mouse model significantly suppressed tumor growth and tumor angiogenesis, and notably inhibited the formation of spontaneous metastases without affecting vital physiological functions [50]. In a separate study, neutralizing anti-HGF antibodies were first developed by Cao et al. who demonstrated that a minimum of three antibodies, each of which act on different HGF epitopes, were required to block c-Met tyrosine kinase activation and the biological outcomes [51]. Moreover, Burgess et al. have shown that fully humanized monoclonal anti-HGF antibodies effectively suppressed HGF-dependent tumor growth in tumor xenograft mouse model [52]. Another fully human HGF antibody, AMG102, was recently tested for its pharmacokinetics and safety in monkeys and further clinical investigation was warranted [53].

It is recently suggested that MET functions in certain human cancers as “oncogene addiction”, the concept formulated in the late 1990s, indicating a constant requirement of MET in these tumors [54]. Therefore, targeting the activated c-Met holds a great promise as an anti-cancer therapy at least for certain tumor types. Regarding c-Met tyrosine kinase receptor inhibitors, a set of low molecular weight compounds including PHA-665752, SU11274, and K252a, which are able to compete for the ATP binding and prevent receptor transactivation and recruitment of the downstream effectors, have recently been tested and shown to effectively inhibit the kinase activity and block the subsequent signaling pathways [55–58] . Particularly, PHA-665742 is capable of inhibiting the autophosphorylation of c-Met with a relatively high specificity compared to other tyrosine and serine-threonine kinases [55,59]. In addition, PHA-665752 was shown to induce massive apoptosis in human gastric cancer cell lines that had amplified MET genes, while it did not affect other cell lines without c-Met receptor amplification [59]. Furthermore, Salgia et al. has recently shown that PHA-665752 treatment inhibited tumorigenicity and angiogenesis in a mouse model of lung cancer xenografts [60]. These results strongly support a potential utility of these compounds for a therapeutic application in the future. Designing a drug that binds the extracellular domain of the c-Met receptor and thus impairing receptor dimerization has been considered as another c-Met blocking strategy. Recently, Petrelli et al. showed that a monoclonal antibody, DN30, prevented c-Met activation and abrogated its biological activity [61]. In addition, soluble recombinant Sema proteins or anti-Sema antibodies against the extracellular Sema domain that is involved in ligand binding and receptor dimerization of c-Met have been generated [62]. As expected, they suppressed the downstream signaling triggered by the c-Met receptor even in the presence of HGF. Another alternative strategy for specifically blocking the receptor is a gene silencing technology. Using adenovirus vectors carrying small-interfering RNA targeting c-MET, Shinomiya et al. demonstrated that the siRNA drastically reduced the c-MET gene expression

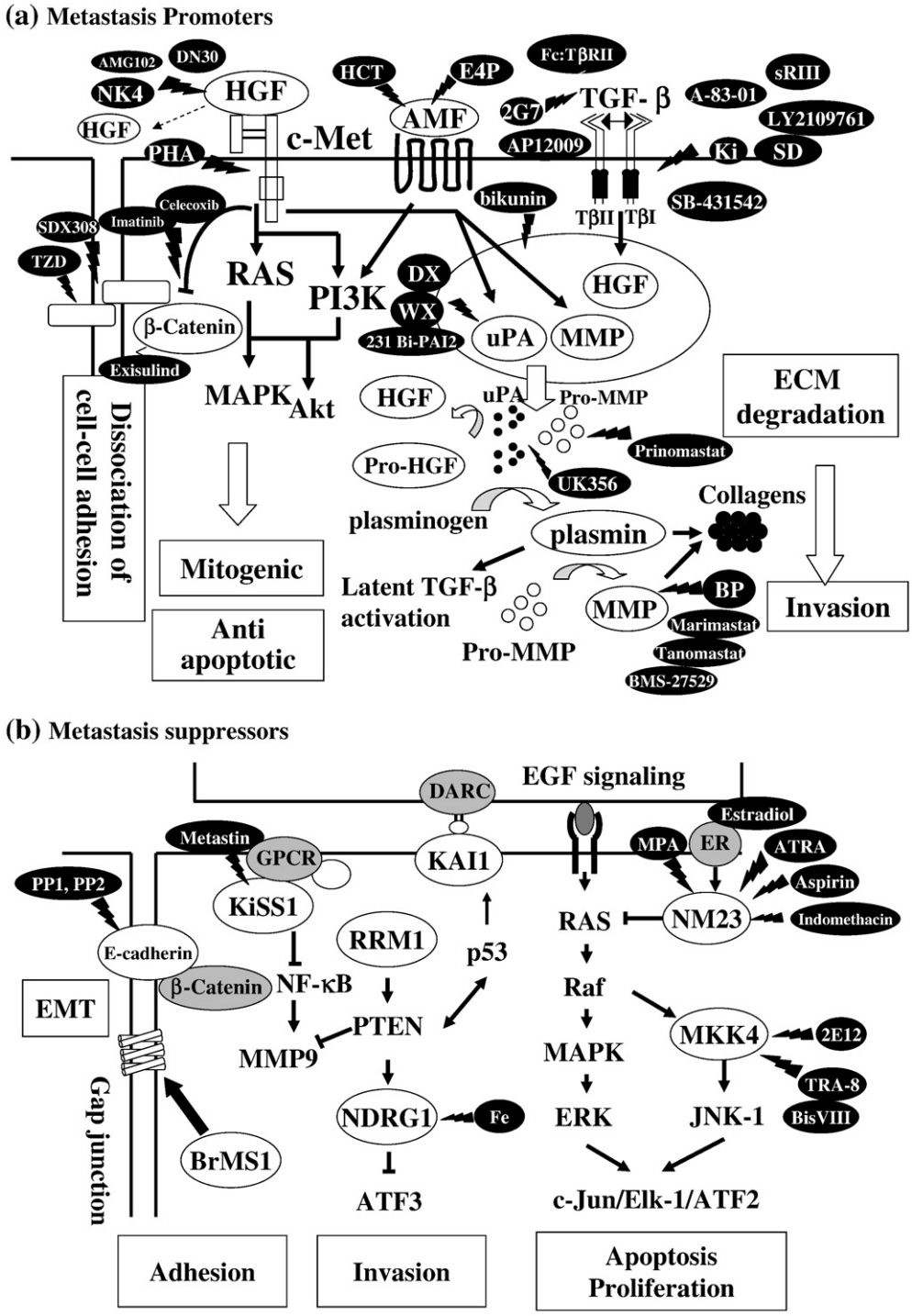

Fig. 2. Signal pathway of tumor metastasis. Tumor metastasis is a result of complex interplay of both positive (a) and negative (b) factors. These pathways and their factors are potential targets for anti-metastatic therapy. The drugs currently under development are shown as black oval shapes.

followed by significant inhibition of proliferation and invasion of various tumor cells lines both *in vitro* and *in vivo* [63]. Collectively, recent information about the mechanistic insight of HGF/c-Met signaling in tumor progression has greatly facilitated the development of a variety of strategies for anti-HGF/cMet therapies, and some of these compounds hold great promises for future clinical application.

3.3. *Tgfβ*

Transforming growth factor-β (TGFβ) is a secreted polypeptide cytokine that plays multiple roles in cell proliferation, differentiation, extracellular matrix production, migration and apoptosis [64-66]. Notably, in normal epithelial cells and at an early stage of tumorigenesis, TGFβ inhibits the proliferation of cells by inducing cell cycle arrest, promoting apoptosis, and enhancing genomic stability [65,66]. However, as the tumor develops, cancer cells become resistant to TGF-β-mediated growth inhibition because of the loss of TGFβ signaling, mutations of cell cycle regulators, or alteration of cross-talk signaling pathways such as activation of Ras [67].

TGFβ1 has been shown to be over-expressed in 74% and 60% of patients with breast and colon cancers, respectively. Interestingly, more intense staining patterns for TGFβ1 are observed in various

types of metastatic cancer including breast, colon, liver, lung, prostate and stomach compared to primary tumors, emphasizing the importance of TGFβ signaling for pro-metastatic activity [68]. Transplanting cell lines stably over-expressing TGFβ1 into athymic mice has been shown to cause increased tumor growth and metastases *in vivo* [69,70]. In another study, transgenic mice that co-express MMTV-Neu and MMTV-TGFβ1 developed mammary tumors with the same latency as the control MMTV-Neu transgenic mice; however the co-transgenics showed significantly more local invasion and elevated numbers of circulating tumor cells and lung metastases [71]. Thus, over-expression of TGFβ can enhance and stimulate tumor growth and malignant progression at least in particular subtypes of tumors. Therefore, TGFβ has been recognized as a tumor promoter at an advanced stage of some tumors, probably by stimulating tumor cell invasion, angiogenesis and immunological surveillance [65,66].

It has been shown that mouse and human carcinomas often over-express TGFβ, which promotes Epithelial-mesenchyma Transition (EMT) via the Smad pathway [66]. Furthermore, Shen et al. have shown that TGFβ was capable of inducing the expression of guanine exchange factor NET1 via Smad3 followed by activation of the Rho GTPase pathway, which results in local disassembly of the actin cytoskeleton and tight junction breakdown [72]. On the other hand, TGFβ can also activate various non-Smad signaling effectors including Ras, Rho GTPase, Erk1/2, PI3K and NF-κB that all play critical roles in EMT, which eventually promotes tumor metastasis [67,73,74]. It has been shown that the motility of metastatic breast carcinoma cells responding to autocrine TGFβ1 did not require Smad activation but rather the activity of the PI3K pathway [74]. In addition, Vogelmann et al. have shown that in polarized epithelial cells, TGFβ blocked cell-cell adhesion by inducing tyrosine phosphorylation of α- and β-Catenin which disrupts the E-cadherin/catenin complexes with actin, and by inducing the expression of transcriptional repressors of the E-cadherin gene such as Snail, Slug and LEF1 [75,76]. Wikstrom et al. showed that the ectopic expression of TGFβ in human prostate cancer correlated with increased angiogenesis around the tumor and eventually lead to a high rate of metastasis of prostate carcinoma cells [77]. The ability of TGFβ to promote angiogenesis is considered to be the action of either inducing expression of VEGF, which directly stimulates the proliferation and migration of endothelial cells, or its chemoattractant activity for monocytes that release angiogenic cytokines [78]. It should be also noted that, in breast cancer, TGFβ stimulates the expression of pTHrP (parathyroid hormone related protein) which promotes osteolytic metastasis and also suppresses late stages of osteoblast differentiation, which leads to net bone loss [79]. Furthermore, TGFβ plays a role in helping tumor cells to escape from the immunological surveillance through its ability to inhibit B and T lymphocyte proliferation and differentiation [80]. TGFβ is also able to deactivate macrophages and thus protect the tumor cells from the immune surveillance [81]. Collectively, because TGFβ often promotes tumor progression in particular subtypes, the components of the TGFβ signaling pathway are being considered as prognostic biomarkers for such tumors as well as potential therapeutic targets [68].

On the contrary, to the tumor-promoting activity of TGFβ, this molecule also has tumor suppressive function at an early stage in some types of cancer. Therefore, TGFβ is considered as a target for chemoprevention for the population with high-risk cancer incidence. To this end, several compounds have been examined and these include FTI-277, Dietary ω-3 fatty acids, Captopril, Suberoylanilide hydroxamic acid (SAHA) and triterpenoids. They are capable of enhancing the expression of TGF receptor (TβRII and TβRI) at mRNA and protein levels, thus increasing the responsiveness of tumor cells to TGFβ with respect to growth arrest and cytostatic effect [82-86]. However, considering the pro-tumorigenic actions of TGFβ, such drugs may have dreadful effects by promoting tumor invasiveness and metastasis. Therefore, current effort is more focused on drugs that block the tumor progression at a later stage. These strategies include developing small molecule inhibitors, affinity- or antibody-based drugs and antisense RNA.

Intense high-throughput screenings have led to the development of selective small molecule inhibitors against the enzymatic activity of the TβRII and TβRI kinases. These inhibitors including SD-208, SD-093, SB-431542, A-83-01 and LY2109761 act as ATP-binding analogues and thus competitively block the catalytic pocket of the receptor kinase [68]. SD-208, an orally active specific TβRI kinase inhibitor, was previously tested in a glioma model, which depends primarily on the pro-tumorigenic action of TGFβ. In this study, SD-208 was found to effectively inhibit the TGFβ-induced glioma cell migration and invasiveness and also to enhance the immunological surveillance [87]. Recently, Reiss et al. also showed that SD-208 treatment resulted in decreased angiogenesis in a mouse model of mammary carcinoma [88]. In addition, Wong et al. showed that SD-208 reduced primary tumor growth and decreased the incidence of metastasis in an orthotopic xenograft mouse model of pancreatic adenocarcinoma [89]. Thus, this inhibitor holds a great promise for future clinical application. Another small molecule for TβRI kinase inhibitor, SD-093, has been shown to strongly decrease the *in vitro* motility and invasiveness of pancreatic carcinoma cells without affecting their growth [90]. Another set of TβRI inhibitors, SB-431542, A-83-01 and LY2109761, all potently affect TGFβ-dependent transcriptional activation and inhibit TGFβ-induced EMT [73]. Interestingly, SB-431542 was demonstrated to reduce colony formation of human lung adenocarcinoma cells, which are growth-dependent on TGFβ; however, it also induced anchorage independent growth of human colon adenocarcinoma cells whose proliferation is promoted by TGFβ [91]. Furthermore, SB-431542 showed no effect on a cell line that failed to respond to TGFβ, which further strengthens the rationale in using this compound as a therapeutic agent of human cancer responsive to tumor-promoting effects of TGFβ. A-83-01 is structurally similar to SB-431542 while it has shown even more potent effect of suppressing TβRI [73]. LY2109761 is a specific pharmacologic inhibitor of TβRI and TβRII kinases. It was demonstrated that this drug was capable of inducing the expression of the Coxsackie and adenovirus receptor (CAR), a tight junction component whose expression is required to be down-regulated for EMT [92]. Currently, some of the above-mentioned specific inhibitors of TβRI have already entered the phase I clinical trials for various human cancers (Table 1).

Neutralizing anti-TGFβ antibodies and the soluble extracellular domain of TβRII with receptor-binding activity have also been pursued as anti-TGFβ approaches. Interestingly, the results of pre-clinical studies have shown that these drugs had a weak and transiently negative effect on primary tumor growth but strongly suppressed metastasis [73]. Pietenpol et al. have demonstrated that the neutralizing antibody 2G7 which has high affinity to three mammalian isoforms of TGFβ showed moderate inhibitory effect on the growth of the primary tumor in an animal model of MDA-MB-231 xenograft, while it almost completely blocked the abdominal and lung metastasis [69]. In addition, enforced expression of the extracellular domain of TβRII has been demonstrated to enhance tumor immune surveillance and strongly inhibit metastasis in animal models of human pancreatic carcinoma [93]. These observations led to a development of a fusion protein of immunoglobulin Fc fragment with the soluble extracellular domain of TβRII (Fc: TβRII) as a therapeutic approach [94]. When tested in vitro, this fusion protein indeed effectively induced apoptosis and inhibited migration of breast cancer cells. Furthermore, Wakefield et al. found that when Fc:TβRII was expressed in the mammary gland of MMTV-based transgenic mouse model followed by a challenge of melanoma cells or by crossing it to the MMTV-Neu mouse, it completely blocked lung metastasis without any adverse side effect [95]. The clinical potential of this experiment is significant especially because the chronic presence of

Fc:TβRII did not show obvious adverse effects. Similarly, Sun et al. have shown that over-expression of soluble extracellular domain of β-glycan (sRIII) antagonized TGFβ in the breast carcinoma cells, which resulted in significant inhibition of metastasis of the tumor cells to the lung, while it moderately blocked the tumorigenic ability [96].

Finally, the antisense DNA or RNAi technology have recently brought a promising development in anti-TGFβ therapy. The oligonucleotide AP12009, which is directed against human TGFβ2, has been tested by administering into brain tumors with continuous infusion and showed better survival time after recurrence than other current chemotherapy against gliomas [97]. Also, RNAi for both TGFβ1 and TGFβ2 in human glioblastoma has been reported to be effective in restoring the proper immune response, which significantly decreased the glioma cell motility and invasiveness [98]. Further investigations in this research field are expected to provide valuable information to improve the efficacy of these compounds and to develop a better delivery system for eventual clinical use of anti-TGFβ therapy.

3.4. Mmp

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, was originally identified to have roles in ECM disruption and thus associated with invasion and metastasis in late stages of cancer progression (Fig. 2A). Years of intense investigations of MMPs have highlighted the significance of these molecules in cancer. MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer, suppresses tumor cell apoptosis, and enhances angiogenesis as well as impairs the host immunological surveillance [99]. Several studies have indicated that cleavage of particular substrates such as insulin-like growth factor binding proteins (IGFBPs) and TGFβ by MMPs can have direct effects on tumor growth [100,101]. In transgenic animals, over-expression of certain MMPs such as MMP1 and MMP3 was sufficient to generate fully malignant tumors in the absence of specific carcinogens [102,103]. In the normal cells or at an early stage of tumor, MMPs can target substrates that influence the apoptotic process of the cells, which is also linked to the chemotherapeutic resistance. Particularly, MMP7 is able to release a soluble form of the death protein Fas Ligand (FasL), which has lower death-promoting potency than the membrane anchored form but has more flexibility to interact with its cognate receptor Fas [104,105]. Thus, the weak but constant apoptotic signal acts as a selective pressure for tumor cells that have elevated anti-apoptotic signals and those that have propensity to acquire additional mutations, which further promote tumor progression. This mechanism is also considered to be the basis of induction of chemoresistance to certain types of tumors [106].

MMPs also play critical roles in angiogenesis. Angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF are usually localized in the matrix and cannot interact with their receptors until freed by MMPs, particularly by MMP9 through ECM proteolysis [107,108]. In addition, MMP9, when recruited to the tumor cell surface and interact with the docking receptor CD44, can proteolytically cleave latent TGFβ and thus promote tumor invasion and angiogenesis [100]. Furthermore, an elegant work of Hanahan and Coussens has shown that MMP9 is predominantly expressed in the tumor-associated stromal cells as well as in macrophages, neutrophils, mast cells and endothelial cells rather than in tumor cells themselves in many cases, which regulates the vascular formation and architecture [109–111]. Intriguingly, Hiratsuka and colleagues have recently shown that MMP9 plays a role in priming premetastatic sites for primary tumor. They demonstrated that tumor-associated macrophages (TAM) induced MMP9 in endothelial cells and in TAMs, which facilitated tumor cell invasion and also prepared the lung as premetastatic niche for the growth of tumor cells in a manner dependent on VEGFR-1 [112].

Escaping from host immune response is a significant problem associated with many cancers. Some MMPs alter the behavior of chemokines and cytokines by specific proteolytic cleavage. For example, MMP9 can suppress the development and propagation of T lymphocytes by disrupting IL-2Rα signaling, resulting in attenuation of a T cell-mediated anti-tumor response [113]. Likewise, CXCL12, also known as SDF1 has been identified as a substrate of MMP2. MMP2-mediated cleavage renders CXCL12 unable to bind its receptor CXCR4, which consequently influence the metastatic dissemination of tumor cells [114].

The strong correlations between altered expression of MMPs at mRNA and protein levels in different human cancers with poor disease prognosis have been well established [99,115]. The over-expression of many MMPs, including MMP-1,-2,-7,-9,-13,-14, is positively associated with tumor progression and metastasis [115]. On the other hand, human breast tumor cells with reduced expression of MMP-8 were found to acquire the metastatic ability compared to their non-metastatic counterpart [116]. Interestingly, Balbín et al. has revealed that MMP8-null mice exhibit an increased tumor susceptibility compared to the wild type because of the attenuation of adaptive immune responses due to the loss of MMP8 [117]. Similarly, MMP-3 knockout mice exhibited increased rate of initial skin tumor growth [118]. However, altered expression pattern or levels of individual MMPs in tumor or stromal cells do not always correlate in the primary tumors and secondary metastatic sites [115]. Interestingly, over-expression of MMPs is frequently accompanied with a corresponding increased expression of natural inhibitors (TIMPs) of MMPs, which result in reduced tumorigenesis in some model systems but does not necessarily inhibit metastasis [119,120]. These discrepancies point out the complexity of MMP functions *in vivo*.

The link between MMPs activity and malignant progression has stimulated serious effort in developing pharmacological inhibitors of MMPs (known as MMPIs) as a potential therapeutic modality since the 1980s [121]. A variety of MMP inhibitors including Marimastat (BB-2516), Prinomastat (AG3340), Tanomastat(BAY12-9566) and BMS-275291 Neovastat were found to be orally active and achieved effective blood levels and displayed high specificity to MMPs while sparing most other types of proteases [122]. These MMPIs have been shown to be effective in controlling cancer progression in animals. However, most clinical trials have come to a crashing halt with the repeated failure in multiple large-scale phase III stage [122]. Even worse, some compounds caused severe side effects such as inflammation, musculoskeletal pain and joint stricture [122]. Considering the ability of MMPs to cleave not only ECM but also a variety of other factors, cytokine precursors and chemokines, it may not be surprising to see unwanted chaotic immune responses. Therefore, this area of research requires newer strategies.

A recent work of Taketo and colleagues has provided valuable insights regarding a possibility of targeting the MMP-producing cell instead of inhibiting MMPs themselves [123]. They found that immature myeloid cells expressing CC chemokine receptor (CCR1), MMP2 and MMP9 infiltrated the tumor invasion front and migrated toward the CCR1 ligand CCL9, whereas blocking CCR1 expression resulted in the accumulation of MMP-expressing cells at the invasion front and suppressed tumor invasion in an animal model. Although an application of this “cellular target” concept is still premature and is waiting to be confirmed by multiple studies, it is expected to cause fewer side effects than the systemic “molecular target” therapy using MMP inhibitors. One important lesson we learned from the past clinical trials of MMPs inhibitors is the need for attention to the stage and type of cancer and the critical selectivity of MMPs inhibitors since the expression pattern of MMPs varies in various cancer types and stages [122]. For example, small cell lung cancer is known to over-express MMP11 and MMP14 rather than MMP2, thus the MMP2 specific inhibitors like Tanomastat and Prinomastat would lead to a poor outcome [124]. One possible strategy is to take advantage of both

the frequent over-expression of MMPs in malignant tumors and the catalytic functions of these enzymes, and this strategy led to the development of protease-activatable retroviral vectors, which contain engineered MMP-cleavable linkers [125,126]. Another approach is to employ macromolecular carriers that are linked to anti-cancer drugs or immune response-stimulating drugs that can be released from its carrier when encountered with MMPs in the tumor environment [127,128]. Alternatively, designing an inhibitor which targets substrate-specific binding sites of MMPs resulting in reduced binding and cleavage of specific substrates of the corresponding MMP opened a possibility of blocking the unwanted catalytic activity of MMPs during tumor progression [99]. Finally, re-screening for MMPs inhibitors from the current anti-cancer drug pool may be worth a consideration. Notably, Bisphosphonates (BP), a class of pyrophosphate analogues widely used in the treatment of breast cancer patients with osteolytic tumors for the past 20 years, was found to significantly inhibit proteolytic activity of MMPs without reducing the expression of MMPs [129]. Although past efforts in developing anti-MMP drugs have been less fruitful than expected, there are still strong rationales and hopes to continue this line of research using more innovative approaches.

3.5. Upa

The urinary-type plasminogen activator (uPA) is a serine protease and able to proteolytically degrade various ECM components and the basement membrane around the primary tumors. It also activates multiple growth factors and MMPs that further contribute to the degradation of the ECM, and thus facilitates tumor cell invasion and intravasation (Fig. 2) [130,131]. Interestingly, a newly identified metastasis suppressor, p75 neurotrophin receptor ($p75^{NTR}$), has recently been demonstrated to suppress metastasis in part by down-regulating specific proteases such as uPA [132]. uPA is produced and secreted as a zymogen (pro-uPA) which binds to the cell surface uPA receptor, uPAR. The pro-uPA is then cleaved by plasmin to become an active form of uPA, which has plasminogen-activating property to convert plasminogen to the active matrix-degrading serine protease plasmin [131]. The proteolytic activity of uPA is regulated by the serine protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2. PAI-1 is able to react with uPA/uPAR-complex and induces internalization of the complex, which results in the intracellular degradation of uPA and PAI-1. On the other hand, PAI-2 forms a complex with uPA and uPAR without internalization, and it is degraded once bound to uPA/uPAR [133]. Because the activity of uPA is dependent on its binding to uPAR, this receptor is also considered to play a crucial role in metastasis [130]. Besides the role in proteolysis, uPAR can interact with and regulate other cell surface proteins such as integrins, growth factor receptors and G-protein coupled receptors to exert its biological functions including chemotaxis, cell migration and invasion, adhesion, proliferation and angiogenesis [134].

Several recent studies have shown that uPAR is also involved in activation of the signaling of other metastasis-promoting factors such as basic fibroblast growth factor (bFGF), VEGF, TGFβ and HGF (Fig. 2) [130,135,136]. Most normal tissues have little or no detectable uPAR, while uPAR is over-expressed across a variety of carcinomas including colon, breast, ovary, lung, kidney, liver, stomach, bladder, endometrium and bone [131,137,138]. uPAR expression has also been shown to be strongly correlated with advanced metastatic cancer, and it is typically found to be abundant at the invasive boundary between tumor cells and normal tissue [139,140]. This localization of uPAR expression in the invasion front may be due to the fact that uPAR is a hypoxia-inducible gene [141,142]. Importantly, the uPAR expression has been found to correlate with a poor prognosis and mortality of patients with various types of solid tumors [141–143]. Currently, the PAI-1 is considered as one of the most informative prognostic markers in several cancer types and a high PAI-1 level is significantly associated with a poor prognosis in these cancers [144–147]. The precise role of PAI-1 in tumor growth and metastasis is yet to be elucidated, but PAI-1 shows diverse functions depending on the cell context and the expression level [148]. Interestingly, several reports indicated that unlike PAI-1, PAI-2 functions as a tumor suppressor and blocks metastasis, and therefore, is associated with a favorable outcome in patients [143,149]. In addition, uPA and PAI-1 have also been reported to be associated with resistance to hormone therapy in advanced breast cancer [150]. Therefore, uPA/PAI-1 can also be used to predict resistance to specific therapies for breast cancer patients. These studies of uPA/uPAR and PAI-1 so far indicate the critical roles of these molecules in tumor progression, suggesting that these proteins serve as excellent therapeutic targets for cancer patients.

In the past, various approaches have been developed to inhibit uPA and its signals. WX-UK1 and WX-671, synthetic serine protease inhibitors developed by WILEX, are the first inhibitors of uPA in world wide clinical trials. Both of them have shown to effectively block metastasis formation and to reduce primary tumor growth in pre-clinical studies, and they have already entered the phase I/II clinical trials as a single agent and/or in combination with other chemotherapeutics for the treatment of patients with metastatic tumors [148]. Bikunin, a Kunitz-type protease inhibitor, is discovered as a potent and selective inhibitor for trypsin and plasmin, while it is moderately effective in inhibiting the catalytic activity of uPA [151]. Kobayashi et al. have also shown that Bikunin was able to down-regulate the expression of uPA and uPAR [152]. Furthermore, Bikunin has been shown to inhibit MAPK and PI3K/Akt signaling, and to effectively inhibit growth and invasiveness of several types of tumor cells [153–155]. Recently, the possibility of using Bikunin as oral therapy was examined in an ovarian cancer model in animal. Results of these experiments have shown that once-daily oral administration of Bikunin had no significant side effects and strongly suppressed the expression of uPA and uPAR, suggesting a utility of Bikunin for an anti-metastatic therapy in humans [156].

DX-1000, another Kunitz domain-based inhibitor of plasmin with specificity, has been previously shown to block tumor growth and metastases *in vivo* with few side effects [157] However, DX-1000 has a quick clearance and short half-life in circulation that challenges the practical utility of this compound in patients. To circumvent these problems, Henderikx et al. conjugated the DX-1000 with polyethyleneglycol (PEG) to prolong *in vivo* half-life. The PEG-conjugated DX-1000 was indeed shown to be effective *in vitro* and significantly blocked tumor proliferation, vascularization and metastasis *in vivo* [158]. More recently, Fishe et al. have shown that 1-Isoquinolinylguanidines (UK-356,202) and its derivatives were able to reversibly inhibit uPA enzymatic activity with selectivity over tPA and plasmin, and it has been selected as a candidate for clinical evaluation [159]. There are also several other strategies currently under active investigation and these include receptor ligand analogues to interfere with the cellular uPA/uPAR interaction, antibodies for PAI-1 and recombinant PAI-2 (231Bi-PAI2) [160–162].

3.6. β-catenin

β-Catenin is an essential component of the cadherin–catenin complex and plays a critical role in the Wnt signaling pathway [163]. The product of the tumor suppressor gene APC (adenomatous polyposis coli) forms a complex with axin/axil, protein phosphatase 2A (PP2A) and glycogen synthase kinase3β (GSK3β) which leads to phosphorylation of β-Catenin thereby inducing degradation of this protein by ubiquitination-mediated proteasomes [164]. The abnormally activated Wnt signaling due to the mutations of APC results in accumulation of β-Catenin followed by promotion of tumorigenesis. Phosphorylation of β-Catenin also releases E-cadherin, which initiates tumor cell migration and tumor metastasis [165,166]. On the other

hand, β-Catenin together with other proteins such as TCF/LEF complex, Reptin and p50, acts as a transcription factor to regulate metastasis-related gene including MMP-9 and KAI1 [167]. More recently, it has been reported that accumulated β-Catenin binds specifically to androgen receptor (AR) and augments the ligand-independent activity of AR in hormone-refractory prostate cancer [168]. Indeed, aberrant expression of β-Catenin has been reported in many types of cancer including colon, bladder, breast, prostate, lung cancer and adrenocortical adenomas [169]. Furthermore, the Wnt/β-Catenin signaling pathway has been shown to be involved in the self-renewal of embryonic stem cells and perhaps in progression of tumor stem cells [170]. Several agents targeting the Wnt/β-Catenin pathway including Exisulind and Imatinib have been shown to inhibit self-renewal of cancer stem cells with varying levels of success [171]. Therefore, targeting β-Catenin and blocking APC/β-Catenin/TCF signals is considered to be a rational approach for developing new anti-cancer drugs.

Exisulind (Aptosyn) and two analogs CP461, CP248 belong to a new class of compounds of SAANDs (Selective Apoptotic Antineoplastic Drugs), which are oxidative metabolites of the nonsteroidal anti-inflammatory drug (NSAID) sulindac. These drugs reduce β-Catenin activity and block Cyclin D1 followed by an induction of apoptosis and inhibition of tumor cell growth [164,172,173]. Currently, Exisulind is in Phase III clinical trials in combination with several chemotherapeutic agents [174,175]. Imatinib (Gleevec), originally identified as an inhibitor of platelet-derived growth factor (PDGF) receptor, has been used in treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and a number of other malignancies. Interestingly, Imatinib has been shown to inhibit tyrosine phosphorylation of β-Catenin, which otherwise releases E-cadherin and promotes cell migration and tumor metastasis [176]. Other strategies including RNAi, antisenseDNA and small molecule inhibitors for blocking β-Catenin have been developed [171,177]. The antisense approach has been used in colon and esophageal cancers as well as leukemia and lymphoma *in vitro*, which lead to reduction of β-Catenin expression and subsequent decrease in the expression of its downstream targets such as Cyclin D1 [177-179].

NSAIDS are also found to be effective in inhibiting the Wnt/β-Catenin signaling pathway. Among them, aspirin and indomethacin were shown to block the transcriptional activity of β-Catenin/TCF [180]. Celecoxib (a COX-2-inhibitor) blocked β-Catenin activity by inducing its degradation via GSK3 β and APC, leading to diminished tumor cell proliferation and survival [181]. R-Etodolac (an enantiomer of Etodolac) and its analog (SDX-308) have been shown to be able to decrease total and activated forms of β-Catenin via GSK3 β activation [182]. These drugs also increased β-Catenin and E-cadherin complex at the membrane site and inhibited β-Catenin-dependent TCF activity followed by decreasing the level of downstream target gene products, Cyclin-D1 and glutamine synthetase [183,184]. In addition to these efforts of directly blocking the β-Catenin activity, selective disruption of β-Catenin-TCF complex and reversing the localization of β-Catenin from cytoplasmic membrane to the nucleus are also considered to be effective approaches for anti-cancer therapy. Thiazolidinedione (TZD), a peroxisome proliferator-activated receptor-gamma ligand, has been demonstrated to completely inhibit lymph node and lung metastases in a xenograft animal model by promoting localization shift of β-Catenin from the nucleus to plasma membrane [185]. TZD also reduced tyrosine phosphorylation of β-Catenin and promoted enhanced expression of E-cadherin [185]. Recently, a crystal structure of β-Catenin-TCF complex has been clarified which shed new light on the molecular mechanism by which this stable and potent transcription factor complex forms [186–188]. Therefore, developing a drug which can disrupt the β-Catenin-TCF complex holds great promise, although how to effectively and selectively disrupt the complex without affecting β-Catenin-E-cadherin or APC complex is still a challenge.

4. Metastasis suppressors

4.1. Nm23

NM23 is the first identified metastasis-suppressor gene in this group. It is located on chromosome 17q21 and codes for an 18.5-kDa protein containing 166 amino acids which functions as nucleoside diphosphate kinase and protein-histidine kinase [189,190]. Clinically, NM23 has been shown to be down-regulated in a variety of tumors including breast and prostate cancers [191,192]. Ectopic expression of NM23 has also been shown to significantly reduce the *in vitro* and *in vivo* metastatic potential of highly metastatic carcinoma cell lines including breast, melanoma, colon, and oral squamous cells [190,193–195]. Recently, Hartsough et al. reported that NM23 formed a complex with Kinase suppressor of Ras1 (KSR1) and phosphorylated this protein at Ser-392 and Ser-434, which resulted in blockade of Ras/MAPK pathway (Fig. 2b) [196]. More recently, Salerno et al. have shown that the NM23 expression level influenced the binding properties, stability and function of the KSR1 in breast carcinoma cells [197]. Hence, NM23 was hypothesized to inhibit MAPK/ERK activation via altering the scaffold function of KSR1 (Fig. 2b). Consistent with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced MAP kinase activity and cell motility *in vitro* as well as diminished incidence of metastasis *in vivo* [196,198,199]. Therefore, NM23 acts as a metastasis suppressor by inhibiting the MAP kinase pathway through the interaction with the KSR1 scaffold protein.

In an attempt to restore the expression of NM23 in tumor cells, several drugs have been found in the past. Among them, medroxyprogesterone acetate (MPA) and estradiol were reported to suppress metastasis through up-regulation of the NM23 gene (Table 1). Medroxyprogesterone is a progestin and commonly used as a component of hormonal contraceptives. Progesterone binds to the progesterone receptor which is then transferred to the nucleus and acts as a transcription factor by binding to the progesterone response elements (PRE) in the promoter region of target genes. Progesterone receptor is known to directly regulate the expression of Cyclin D1, beta-casein and $p21^{WAF1}$ as well as MAPK [200–205]. MPA has a long history of clinical use at a low dose as the contraceptive Depo-Provera and has also been used for hormone replacement therapy in combination with estrogen [206]. At a high concentration, it has been used for the treatment of advanced breast and endometrial cancers [207]. MPA can competitively bind to several steroid hormones including progesterone (PR), androgen (AR) and glucocorticoids (GR), and thus it is able to up-regulate NM23 by antagonizing the effect of glucocorticoid response element (GRE) on the NM23 promoter [208]. Ouatas et al. previously found that MPA inhibited the soft agar colonization of breast carcinoma cells by up-regulating the NM23 expression [209]. In *in vivo*, Palmieri et al treated mice xenografted with breast carcinoma cells with MPA and found 27–36% reduction of metastasis incidence in the treated animals.

Estradiol works as an estrogen to modulate gene expression via binding to its intracellular receptor ERs [210]. Interestingly, Estradiol was found to be able to decrease the number of experimental lung metastases in nude mice when they were injected with breast cancer cell line MDA-MB231 with forced expression of ER (Table 1) [211]. Lin et al. reported that the level of NM23 mRNA and protein was induced by Estradiol in breast cancer cell lines with the extent that these effects correlated with the level of ERα expression [212]. In addition, Estradiol was shown to be able to decrease the invasive ability of ERα positive carcinoma cell lines MCF7 and BT-474, while it did not have any effect on BCM-1 cell which had virtually no ERα expression [212]. Therefore, it is suggested that Estradiol was able to suppress tumor metastasis by activating the expression of the NM23 gene in an ERα-dependent manner (Fig. 2b) [212].

Many of the therapeutic effects of nonsteroidal anti-inflammatory agent (NSAIDs) are clearly due to the inhibition of prostaglandin synthesis by inactivation of cyclooxygenase 1 and 2 (COX-1 and COX-2) [213]. The anti-tumor effect of NSAID has been recognized when Aspirin was found to reduce the risk of colorectal adenoma and carcinoma in animal models [214–217]. Interestingly, Yu et al. reported that Aspirin decreased the invasive potential of COX2 negative colon cancer cells via up-regulation of NM23 expression (Table 1) [217].

Another NSAID, Indomethacin, was also found to up-regulate the expression of NM23 in breast cancer cells and to alter the malignant choline phospholipid phenotype toward a less malignant tumor [218]. Reich et al. reported that indomethacin reduced the invasive ability of human fibrosarcoma and murine melanoma cell lines and that murine melanoma cells exposed to indomethacin prior to i.v. injection produced significantly fewer lung metastases (Table 1) [219]. Kundu et al. also reported the anti-metastasis effect of indomethacin by oral administration in a murine model [220]. They transplanted a murine mammary adenocarcinoma cell line 410.4 and found that the metastatic ability of this cell line was reduced by almost 50% with the treatment of indomethacin (Table 1) [220]. Therefore, indomethacin has potential utility as an anti-metastatic drug and it is currently under clinical trial.

All-*trans* Retinoic Acid (ATRA) is known as the first successful targeted drug for cancer therapy. ATRA causes the differentiation of leukemic myeloid cells from mature myeloid cells by attaching to one of several retinoid receptors in the cell nucleus and then directly modulating gene expression [221–223]. The down-regulation of several oncogenes including Ras and c-fms by ATRA has been reported [224,225]. Interestingly, the expression of NM23 was also shown to be up-regulated by ATRA in human hepatocarcinoma cell line and gastric cancer cell lines [226,227]. Liu et al. demonstrated that treatment with either ATRA or transfected NM23 cDNA reduced metastasis-associated phenotypes including chemotaxic cell migration and invasion of human hepatocarcinoma cell line [226]. Furthermore, Wu et al. examined the effect of ATRA treatment in xenografted nude mice and found that ATRA treatment significantly decreased the metastasis in liver and increased NM23 protein levels in experimental groups compared with a control group [227]. Since ATRA was also able to reduce cell growth *in vitro* and *in vivo* [227], the specificity of ATRA treatment on tumor metastasis is still unclear. However, a combination treatment of ATRA and IFN-alpha in a clinical trial was well tolerated, and patients who have metastatic osteosarcoma were found to be in stable complete remission 14 months after the end of therapy [228]. Therefore, further investigation of ATRA as an anti-metastatic drug is warranted.

4.2. KiSS-1

KiSS-1 was originally identified as a metastasis-suppressor gene using a combined strategy of MMCT and differential display [229]. The introduction of an intact copy of whole human chromosome 6 into the C8161 human melanoma cell resulted in significant reduction of metastasis ability of this cell line without affecting tumorigenicity or local invasiveness in animals [229]. Later Lee et al. reported that the KiSS-1 gene was actually mapped on chromosome 1q region which is frequently deleted in late-stage human breast carcinomas [230]. They then transfected the KiSS-1 gene into human breast ductal carcinoma cell line MDA-MB-435 and found that KiSS-1 almost completely suppressed metastatic activity of MDA-MB-435 [230]. Therefore, although the KiSS-1 gene is located on chromosome 1, it is believed that chromosome 6 is responsible at least in part for its metastasis suppressive effects by harboring a gene that positively regulates KiSS-1 expression [231]. Clinically, the expression of mRNA of the KiSS-1 gene was found to be significantly down-regulated in metastatic tumors, which is in accordance with the idea that KiSS-1 is a metastasis suppressor [232].

Ectopic expression of the KiSS-1 gene was shown to significantly reduce the rate of three-dimensional growth in soft agar, but it did not affect invasion or motility [230]. These results suggest that KiSS-1 affects downstream of cell-matrix adhesion and perhaps involves cytoskeletal reorganization. On the other hand, Yan et al. reported that KiSS-1 transfected HT1080 cells showed substantially reduced enzyme activity of MMP9 with specific down-regulation of mRNA level of MMP9 and invasiveness of tumor cells *in vitro* [233]. They have further shown that this effect was partly attributable to the ability of KiSS-1 to reduce NF-kB binding to the promoter of MMP9 by enhancing I-kB activity (Fig. 2b) [233].

Metastin is a 54 amino acid peptide whose sequence is identical to a part of the KiSS-1 gene, and this peptide was found to act as a ligand for orphan G-protein coupled receptor (hOT7T175, AXOR12, GPR54) (Table 1) [234,235]. Interestingly, Ohtaki et al. have shown that Metastin significantly attenuated pulmonary metastasis in a mouse xenograft model using the B16-BL6MR melanoma cell, while Metastin had no direct effect on the primary tumor growth [234]. Importantly, Metastin was found to be able to suppress the degree of pulmonary metastasis even when the peptide was administered to the mice that already had metastasis in the lung [234]. Therefore, Metastin is considered to be a promising agent for the treatment of metastatic cancer patients. In this regard, it is encouraging that the expression of the Metastin receptor genes was found to be normal even when KiSS-1 was significantly down-regulated in various types of cancers [236]. These results suggest that Metastin may be effective even in advanced cancer that has lost KiSS-1 expression.

4.3. Mkk4

Chekmareva et al. has previously demonstrated a prostate cancer metastasis-suppressor activity encoded by a discontinuous ~70 cM region of human chromosome 17, which suppresses the spontaneous metastatic ability of highly metastatic Dunning AT6.1 rat prostate cancer cells [237]. Later, Yoshida et al. identified the MKK4/SEK1 (Mitogen-activated protein kinase kinase 4) gene in this chromosomal region as a candidate metastasis suppressor [238]. Ectopic expression of MKK4 in highly metastatic prostate cancer cell line indeed significantly suppressed macroscopic lung metastasis without affecting the primary tumor growth in animals [238]. Furthermore, Kim et al. examined the status of MKK4 expression in clinical samples of prostate cancer by immunohistochemical analysis and found that the expression of MKK4 was inversely correlated with Gleason score and tumor progression [239]. How MKK4 suppresses metastasis is a crucial question and has been under active investigation. MKK4 belongs to MAP kinase family which plays central roles in cell proliferation, differentiation and apoptosis. It is known that MKK4 is activated in response to a variety of extracellular stimuli including stress followed by activation of JNK(c-Jun N-terminal kinase) and/or p38 MAPK pathways (Fig. 2b) [240]. It is plausible that, when a tumor cell reaches a distant organ site, the expression of MKK gene in cancer cell is suppressed in the stressful environment, and therefore, fails to establish colonization.

A strategy of using monoclonal antibodies has been considered to be an attractive approach for cancer therapy due to their high target specificity. Anti-death receptor antibody such as anti-TRAIL antibodies, 2E12 and TRA-8, have been found to activate the MKK4/JNK/p38 pathway, suggesting a potential utility of the antibodies for anti-metastatic therapy [241]. Furthermore, Ohtsuka et al. reported that the combination of the anti-death receptor antibodies and chemotherapy agents led to a synergistical activation of the JNK/p38 MAP kinase which was mediated by MKK4 (Table 1) [241]. In their studies, agonistic anti-TRAIL antibodies 2E12 and TRA-8, when combined with chemotherapeutic agents such as Adriamycin, were able to increase the release of cytochrome c and Smac/DIABLO from mitochondria in parallel with the profound loss of mitochondrial membrane potential, which resulted in apoptosis in breast, prostate and colon cancer cells

[241]. It is interesting to test whether these regimens are able to suppress metastatic potential of MKK-positive cancer cells *in vivo*. Bisindolylmaleimide VIII was originally developed as a synthetic inhibitor of protein kinase C (PKC) [242,243], and it was later found to promote Fas-mediated apoptosis in a PKC-independent manner [244]. Ohtsuka et al. examined a possible effect of Bisindolylmaleimide VIII on TRA-8 induced apoptosis and found that a combination of Bisindolylmaleimide VIII and TRA-8 induced 50–80% of apoptosis in human astrocytoma cell line (1321N1), while the treatment of the cells with TRA-8 alone induced apoptosis only in up to 20% of the cells [245]. In *in vivo*, either Bisindolylmaleimide VIII or TRA-8 alone partially regressed the xenografted tumor in NOD/SCID mice, while the combination of these two drugs almost completely blocked the tumor growth. However, whether Bisindolylmaleimide VIII enhances TRA-8- induced apoptosis via a role in regulating MKK4/JNK/p38 apoptosis kinase signaling and whether the combination of these drugs indeed suppresses metastasis remains to be examined.

4.4. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screening a cDNA library of the human liver [246]. The E-cadherin is a calcium-dependent adhesion molecule which constitutes the adherence junction in epithelial cells [247,248]. Reduced level of E-cadherin is shown in a variety of human cancers at advanced stages. It is believed that a low level of E-cadherin can give advantage to tumor cells on breaking the adhesion junction and detaching from adjacent cells, so that these cells invade and metastasize to other distant organs. Clinically, several groups have reported that decreased expression of E-cadherin was associated with a poor prognosis in cancer patients [249]. On the other hand, over-expression of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness [250]. In addition, using a transgenic mouse model of pancreatic β-cell carcinogenesis (Rip1Tag), Perl et al. showed that tumor incidence or tumor volume was not significantly changed between double-transgenic Rip1Tag2xRip1dnE-cad mice and single-transgenic Rip1Tag2 littermates [251]. However, the double-transgenic mouse developed metastases to the pancreatic lymph nodes, an invasive phenotype that was never observed in single-transgenic Rip1Tag2 mice [251]. Therefore, E-cadherin is considered to function as a metastasis suppressor. Generally, E-cadherin plays an important role in epithelial-mesenchymal transition (EMT) during which epithelial cells lose their cell-cell junctions and acquire mesenchymal characteristics to endow the migratory ability to tumor cells [249]. E-cadherin interacts with β-Catenin to mediate actin binding (Fig. 2b) [252]. Therefore, loss of E-cadherin, in addition to reducing cell-cell adhesion, provides an oncogenic stimulus by freeing β-Catenin from the membrane, so that β-Catenin can travel to the nucleus to activate TCF-regulated genes such as c-Myc and Cyclin D1 [253]. Furthermore, E-cadherin has been recently found to be down-regulated by transcription factors Snail and Slug that are involved in the process of EMT, cell differentiation and apoptosis [254]. Therefore, restoring the function of E-cadherin is considered to be a potential therapeutic option for metastatic disease. PP (pyrazolo [3,4-d] pyrimidines)1and PP2 were originally identified as selective inhibitors for Src, and they were shown to be able to block tumor growth and to reduce metastasis in a mouse pancreatic model. However, these compounds have also been found to reactivate the E-cadherin expression in pancreatic and colon cancer cells (Table 1) [255,256]. Therefore, PP1 and PP2 may serve as effective anti-metastatic drugs although they need to be tested more extensively in a clinical trial.

4.5. Ndrg1

N-myc downstream regulated gene 1 (NDRG1) was originally identified by differential displays as being significantly up-regulated by induction of *in vitro* differentiation of colon carcinoma cells [257]. The protein encoded by the NDRG1 gene has a molecular weight of 43 kDa and possesses three unique 10-amino acids tandem repeats at the C-terminal , among which seven or more phosphorylation sites were predicted and later they were shown to be targets of protein kinase A *in vitro* [258]. The NDRG1 gene is controlled by multiple factors and responsive to various stimuli. The expression of NDRG1 was repressed by C-myc and N-myc/Max complex *in vitro*, while it was induced by p53, hypoxia and PTEN (Fig. 2b) [259]. NDRG1 has been shown to act as a tumor suppressor as well as a tumor metastasis suppressor depending on cell context [259]. In a clinical setting, NDRG1 was found to be consistently expressed in normal prostate tissue as well as PIN (prostatic intraepithelial neoplasia) and BPH (benign prostatic hyperplasia), whereas the expression was significantly reduced in high-grade tumors [260,261]. In addition, the level of the NDRG1 expression was inversely co-related with the status of metastasis in these patients, supporting the notion that NDRG1 is a tumor metastasis suppressor [260]. In breast cancer, a similar and significant negative correlation of NDRG1 with metastasis has been observed, while the expression of NDRG1 does not show any significant correlation with the size or the histological grade of the primary tumor [261]. These results strongly suggest the negative involvement of NDRG1 in the process of invasion and metastasis in both prostate and breast cancer. Furthermore, ectopic expression of the NDRG1 gene in a highly metastatic prostate cancer cell line significantly reduced the incidence of lung metastases, suggesting that NDRG1 was able to block the metastatic process without affecting the primary tumor growth [260,261]. Similar metastasis suppressor effect of NDRG1 was also observed in colon carcinoma cells by Guan et al. [262]. In addition, NDRG1 also significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay [260,261]. Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that NDRG1 is a metastasis-suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How NDRG1 suppresses the tumor metastasis is an intriguing question which is under active investigation.

Recently, Fe chelators, desferrioxamine (DFO) and 311 were shown to be able to up-regulate the NDRG1 expression in human breast cancer cell line MCF7 [263]. In the past years, dietary Fe restriction has been shown to markedly decrease tumor growth in rodents [264–266], and Fe chelators such as Triapine and desferrioxamine (DFO) were reported to be potentially useful for cancer therapy (Table 1) [266–268]. More recently, Whitnall et al. examined the effect of another Fe chelator, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT), on tumorigenesis in xenografted mice models of lung carcinoma, neuroepithelioma and melanoma and found that Dp44mT strongly inhibited the growth of all tested human xenografts in nude mice [269]. Notably, Dp44mT significantly augmented the expression of the NDRG1 gene in the tumor compared to that of control group, suggesting a promising utility of this compound as an anti-cancer as well as anti-metastatic drug [269].

5. Conclusion and future direction

Despite significant improvement in surgical techniques and chemotherapy for cancer treatment in general, none of the current medical technologies “cure” the metastatic disease, and the patients who have already acquired metastatic cancer are left virtually with no options. Therefore, there is an urgent need for developing a novel approach of target-specific therapy to metastatic tumor cells, which requires more comprehensive understanding of the molecular mechanism of metastases. The goals of anti-metastatic therapy are three folds. Firstly, we need to develop a specific drug that blocks secondary metastasis to treat patients who have already acquired metastatic disease but are still at an early stage. Secondly, a drug

should also be developed to treat patients who underwent surgical resection of their primary tumors in order to prevent a possible recurrent disease. However, the ultimate goal is to develop a non-toxic agent which can be taken as diet for prevention of metastasis. In the past decade, the major effort of anti-cancer research has been focused on the development of drugs that can block the proliferation of tumor cells. They take advantage of the fact that tumor cells are more actively proliferating than other normal cells, and therefore, "selectively" kill the cancer cells. However, this "selectivity" has narrow margins and these agents inevitably cause severe side effects even when they are used in combination to lower the toxicity. From these experiences, we have learned an important lesson that the most critical issue for anti-cancer drugs is their specificities. Therefore, to develop an anti-metastatic drug, it is crucial to define a target molecule which is specifically expressed in metastatic cells. Ideally, an agent which can attack the molecule is inactive (pro-drug) when given to patients, and is activated only in the tumor cells. In theory, monoclonal antibodies and siRNA are highly specific to target genes, and active investigations are underway to utilize these technologies for the development of anti-metastatic drugs. If a target is well defined and specific, these agents are considered to be very effective, although there are still many unknown technical questions such as stability and delivery method of these agents. However, recent advancement of bio-technology such as nano-particles has provided us with a hope that we can eventually overcome these problems.

We have learned a great deal of the metastasis cascade, and many new genes and signal pathways involved in this process have been identified. Some genes hold great promises as potential druggable targets. The genes that control EMT and cell motility as well as their signal pathways are rational candidates for the drug development. Although a clinical trial of the drugs that block MMP resulted in a rather disappointing outcome, these molecules are still considered to be excellent targets. The fact that metastatic cells are the only epithelial cells in circulation may provide us with a window of opportunity to attack such cells. In addition, tumor cells are often attracted by various types of chemokines to the distant organ sites, and these chemokines may also serve as molecular targets for anti-metastatic therapy. Reactivation of metastasis-suppressor genes and their signal pathways such as MKK/JNK, PTEN/Akt and NDRG/ATF are also a rational strategy. Recent finding that KAI1 blocks metastasis by inducing senescence upon interaction with endothelial cells also suggests an interesting possibility to develop an effective drug to activate the KAI1 pathway. Perhaps, genome wide shRNA library screening and comprehensive proteomics approach may reveal more suitable targets for metastatic therapy in the near future. The use of computer-driven strategies such as automated determinations of the structures of target molecules and computer-aided design of drug molecules followed by a high-throughput screening has already begun to set this trend into motion.

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Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1

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Abstract

The fatty acid synthase (FAS) gene is significantly up-regulated in various types of cancers, and blocking the FAS expression results in apoptosis of tumor cells. Therefore, FAS is considered to be an attractive target for anticancer therapy. However, the molecular mechanism by which the FAS gene is up-regulated in tumor cells is poorly understood. We found that FAS was significantly up-regulated by hypoxia, which was also accompanied by reactive oxygen species (ROS) generation in human breast cancer cell lines. The FAS expression was also activated by H_2O_2, whereas *N*-acetyl-L-cystein, a ROS inhibitor, suppressed the expression. We also found that the hypoxia significantly up-regulated sterol regulatory–element binding protein (SREBP)-1, the major transcriptional regulator of the FAS gene, via phosphorylation of Akt followed by activation of hypoxia-inducible factor 1 (HIF1). Moreover, our results of reporter assay and chromatin immunoprecipitation analysis indicate that SREBP-1 strongly bound to the SREBP binding site/E-box sequence on the FAS promoter under hypoxia. In our xenograft mouse model, FAS was strongly expressed in the hypoxic regions of the tumor. In addition, our results of immunohistochemical analysis for human breast tumor specimens indicate that the expressions of both FAS and SREBP-1 were colocalized with hypoxic regions in the tumors. Furthermore, we found that hypoxia-induced chemoresistance to cyclophosphamide was partially blocked by a combination of FAS inhibitor and cyclophosphamide. Taken together, our results indicate that FAS gene is up-regulated by hypoxia via activation of the Akt and HIF1 followed by the induction of the SREBP-1 gene, and that hypoxia-induced chemoresistance is partly due to the up-regulation of FAS. [Cancer Res 2008;68(4):1003–11]

Introduction

Fatty acids have long chains of lipid-carboxylic acid and play pivotal roles in normal cellular function as well as in homeostasis of the whole body. They are the source of membrane components, such as phospholipids and glycolipids, and also provide precursors of critical signal molecules for proliferation and differentiation (1). Fatty acids also function as a medium to store energy in the adipose tissue (2). In general, normal adult cells acquire fatty acid mainly from dietary source and rarely use the pathway of *de novo* synthesis, except in the liver, adipose tissue, and lactating mammary gland (3, 4). In striking contrast, many human tumor cells synthesize fatty acids by using the *de novo* pathway as was originally observed by Medes et al. (5) >50 years ago. Fatty acid synthase (FAS) is the major enzyme of lipogenesis and catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce palmitic acid in the presence of NADPH (6). The FAS gene is highly up-regulated in various types of human malignancies, although this gene is expressed at minimum or undetectable level in most normal tissues, and therefore, FAS overexpression is considered to be one of the most common molecular changes in cancer cells (7–11). Importantly, treatment of tumor cells with pharmacologic inhibitors of FAS leads to cell cycle arrest, followed by apoptosis of the tumor cells (12). We have previously shown that specific blocking of the FAS expression by using siRNA in breast cancer cells caused an accumulation of malonyl-CoA, which led to the inhibition of carnitine palmitoyl transferase-1 as well as up-regulation of ceramide (13). This was also followed by the induction of the proapoptotic genes, BNIP3, TRAIL, and DAP kinase 2, which resulted in the apoptosis of the tumor cells (13). These observations suggest that FAS overexpression confers selective advantage to tumor cells by inhibiting apoptosis and promoting cell cycle progression.

How the FAS gene is up-regulated in cancer cells is an intriguing question, although it has been poorly understood. FAS was previously found to be up-regulated by several growth factors and their receptors including epidermal growth factor, Her2 (ErbB2/neu), and keratinocyte growth factor (14–16). Upon binding to each receptor, these factors transmit cellular signals such as mitogen-activated protein kinases (Erk1/2 MAPK2), Janus kinase (JNK), and phosphotidylinositol 3′-kinase (PI3K) followed by Akt activation (17–20). The activation of Akt is commonly observed in a variety of tumors and seems to contribute to the up-regulation of the lipogenic enzymes. On the other hand, a lack of expression or mutation of the tumor suppressor gene, PTEN, has been well established in various types of tumors, and PTEN blocks the function of Akt by counteracting PI3K through dephosphorylation of this enzyme (21). In fact, we have recently shown that the expression of PTEN has a significant inverse correlation with FAS expression in prostate cancer patients (22), and that the inhibition of the PTEN gene expression *in vitro* indeed led to the overexpression of FAS, although ectopic expression of PTEN significantly suppressed FAS (22). On the other hand, sterol regulatory–element binding protein (SREBP) have been known to be the key transcription factors to regulate lipogenic genes, and the FAS gene was indeed shown to be significantly activated by SREBP-1. Interestingly, Porstmann et al. (20) recently found that Akt stimulated the synthesis and nuclear localization of activated

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SREBP-1 followed by activation of the FAS gene. Therefore, PTEN-Akt pathway and the downstream effectors play a critical role in the FAS gene regulation in cancer cells. Furthermore, FAS expression has also been found to be controlled by tumor suppressors and oncogenes, including p53, p63, p73, and H-ras (18, 23). Therefore, overexpression of FAS is often associated with phenotypic changes of cell transformation that are induced either by oncogenes or tumor suppressors. These observations strongly suggest that FAS overexpression is actively contributing to the process of cell transformation rather than merely a consequence accompanied with the phenotypic changes.

Because FAS alone is not likely to cause cellular transformation but rather provides growth advantage to tumor cells by blocking proapoptotic genes, it is plausible that the FAS gene is up-regulated by tumor microenvironment, such as hypoxia, a hallmark of tumors, as a survival strategy of tumor cells. This assumption is supported by the observation that the Akt pathway is activated by a hypoxic condition, and that Akt is also capable of activating SREBP-1, which is a key transcription factor of the FAS gene. In this article, we tested a possibility of regulation of FAS expression by hypoxia in breast tumor cells both *in vitro* and *in vivo* and found that the FAS gene is indeed up-regulated by hypoxia through induction of Akt followed by activation of hypoxia-inducible factor 1 (HIF1) and SREBP-1. We also found that hypoxia-induced chemoresistance, which is a major clinical obstacle, can be partially overcome by a combination of a FAS inhibitor and a chemotherapeutic drug.

Materials and Methods

Cell culture and reagents. Human breast carcinoma cell lines, MX1, MCF7, MDA-MB231, and MDA-MB157 were purchased from American Type Culture Collection. The cells were maintained in RPMI 1640 supplemented with 10% FBS, streptomycin (100 μg/mL), penicillin (100 units/mL), and 250 nmol/L dexamethasone (Sigma Chemical Co.) and grown at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced with DMEM before hypoxia treatment at 37°C in GasPak (BD Diagnostic Systems). Hydrogen peroxide, *N*-acetyl-L-cysteine (NAC), cerulenin, YC-1, and cyclophosphamide were purchased from Sigma Chemical Co. LY294002 was obtained from Calbiochem. The expression plasmid of Akt1, Addgene plasmid 9008, which expressed activated (myristoylated) form of Akt1, was purchased from Addgene (24). siRNA for Akt and scramble sequence for control were obtained from Cell Signaling. siRNA for siSREBP was purchased from Santa Cruz Biotechnology.

Western blot. The cells were collected and resuspended in lysis buffer [50 mmol/L Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, and 1 mmol/L EDTA]. The lysates were boiled for 5 min, resolved by SDS-PAGE on an 8% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were treated with antibodies against FAS (0.2 μg/mL; Immuno-biological Laboratories Co.), β-tubulin (1:1,000; Upstate Biotechnology), HIF1 (1:200; BD Bioscience), SREBP-1 (1:200; Santa Cruz Biotechnology), phospho-Akt (1:200; Ser^{473}; Cell Signaling), Akt (1:200; Cell Signaling), and phospho-SREBP (0.5 μg/mL; ref. 25). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by ECL Plus system (Amersham Life Sciences).

Quantitative real-time PCR. Total RNA was isolated from the cells and reverse transcribed. The cDNA was then amplified with a pair of forward and reverse primers for the following genes: FAS (5′-CATCCAGATAGGCCTCATAGAC-3′ and 5′-CTCCATGAAGTAGGAGTGGAAG-3′), SREBP (5′-CTGGTCTACCATAAGCTGCAC-3′ and 5′-GACTGGTCTTCACTCTCAATG-3′), and β-actin (5′-TGAGACCTTCAACACCCCAGCCATG-3′ and 5′-CGTAGATGGGCACAGTGTGGGTG-3′). PCR reactions were performed using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR kit (Finnzyme Corp.). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 min followed by 40 cycles of PCR using the following profile: 94°C, 30 s; 63°C, 30 s; and 72°C, 30 s.

Reactive oxygen species assay. The cells were cultured in RPMI medium and fluorophore dichlorodihydrofluorescein diacetate (DCFDA; Sigma Chemical Co.) was added directly to the medium at a final concentration of 50 μmol/L. The culture was further incubated at 37°C for 1 h, and the cells were washed with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by the MCID software.

Reporter assay. To generate the reporter plasmid for chloramphenicol acetyl transferase (CAT) reporter assay, the promoter region of FAS (from +4 to −1,328 bp) was amplified where the forward primer included the *Hin*d III linker and reverse primers included *Bgl* II linker. The PCR products were cloned into the pBLCAT3 plasmid. A series of deletions were constructed by Erase-a-Base System (Promega) according to the manufacturer's protocol. These plasmids were transfected to breast cancer cell line MCF7 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were collected and then subjected to CAT assay as described previously (26). The reaction was done, and acetylated [^{14}C]Chloramphenicol was quantified with a PhosphorImager (Packard Instruments). The luciferase reporter plasmid of the FAS promoter was a gift from Dr. Verhoeven (Catholic University of Leuven, Leuven, Belgium; ref. 27). To delete eight bases of the SREBP binding/E-Box sequence on the FAS promoter from this plasmid, QuickChange Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's protocol. The luciferase reporter plasmids were transfected to MCF7 as described above. Luciferase activities were then measured by using Dual-Luciferase Reporter Assay System (Promega) and Luminometer (Berthold Detection Systems). For each transformation experiment, the Renilla expression plasmid phRG-TK (Promega) was cotransfected as an internal control, and promoter activities were normalized accordingly.

Chromatin immunoprecipitation. MCF7 cells were cultured in T75 flask and fixed with 1% formaldehyde for 10 min at room temperature. To stop the reaction, 125 mmol/L glycine was added to the culture medium, and the cells were washed with PBS and harvested. Cells were then suspended in cell lysis buffer (5 mmol/L PIPE, 85 mmol/L KCl, and 0.5% NP40) and homogenized with a type A Dounce homogenizer. The cell nuclei were collected and lysed with Nuclei lysis buffer (50 mmol/L Tris-HCl, 10 mmol/L EDTA, and 1% SDS). The chromatin was sonicated on ice to an average length of 400 bp. The sample was then centrifuged at 4°C, and the precipitates were resuspended in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mmol/L Tris-HCl, 167 mmol/L NaCl, 1.1% Triton X-100, and 0.01% SDS). After preclearing the sample with Protein G agarose beads (Dynal Biotech) followed by brief centrifugation, the supernatant was transferred to a new tube and anti–SREBP-1 (Santa Cruz Biotechnology) antibody was added. After 24 h of incubation at 4°C, Protein G agarose beads were added, and the sample was incubated for 3 h at 4°C. The beads were then washed with washing buffer (100 mmol/L Tris-HCl, 500 mmol/L LiCl, 1% NP40, and 1% deoxycholic acid), and DNA-protein complexes were eluted with elution buffer (100 mmol/L $NaHCO_3$ and 1% SDS). DNA protein was decrosslinked followed by phenol extraction, and the purified DNA was subjected to PCR using both specific (5′-TCATTGGCCTGGGCGGCGCAG-3′ and 5′-AAACCGCGGCCATCCCCGGGC-3′) and nonspecific primers (5′-CAGCCAGAGACACCTGTGGCC-3′ and 5′-CCTTTTCTGACCGCTTCGCGC-3′) for the SREBP binding/E-box sequence of the FAS promoter. The PCR products were visualized after electrophoresis on 8% acrylamide gel followed by staining with ethidium bromide.

Immunohistochemistry. Human breast cancer specimens were obtained from surgical pathology archives of the Akita Red Cross Hospital. All of the tissue sections were obtained by surgical resection. For immunohistochemical staining, 4-μm-thick sections were cut out from the formaldehyde-fixed and paraffin-embedded tissue specimens and mounted on charged glass slides. The sections were baked at 60°C for 1 h, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were heated in 10 mmol/L sodium citrate (pH 6.0) at 85°C for 30 min. The slides were treated with 3% H_2O_2 to block endogenous peroxidase activity and then incubated overnight at 4°C with anti-FAS rabbit polyclonal

antibody (0.2 μg/mL; Immuno-biological Laboratories Co.), anti–SREBP-1 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), or anti–carbonic anhydrase 9 (CA9) mouse monoclonal antibody (1:100; R&D Systems, Inc.). The sections were then incubated with horseradish peroxidase–conjugated anti-rabbit or mouse IgG for 30 min at room temperature, and 3,3′-diaminobenzidine substrate chromogen solution [Envision-plus kit (Dako Corp.) or ABC staining system (Santa Cruz Biotechnology)] was applied. Finally, the sections were counterstained with hematoxylin. Results of the immunohistochemistry were judged based on the intensity of staining, comparing the tumor cells and the normal glands on the same slide. Grading of the FAS, SREBP-1, and CA9 expression levels was done by two independent persons without prior knowledge of the patient data. The cases were then divided into those that showed positive staining and those that showed reduced expression of the two genes.

Animal model. Breast cancer cell line, MDA-MB231, was suspended to 30 million/mL with PBS, and equal volume of Matrigel (BD Biosciences) was mixed with the tissue. The cell suspension (0.1 mL) was injected into the mammary fat pad of 4-week-old female nude mice. A disc of 17B-estradiol (Invitrogen) was also embedded under the skin of these mice. After 3 weeks, 0.2 mL of pimonidazole (Hypoxyprove-1 kit; Chemicon; 2,000 μg/mL) was injected to the mouse via i.p. After 2 h of the injection, the mouse was euthanized and the tumor excised and snap frozen. The tumor sample was cut into a 4-μm slice and mounted on charged glass slides. Immunohistochemical analyses were performed for these slices using anti-pimonidazole and anti-FAS antibodies.

***In situ* apoptosis assay.** The cells were grown in 96-well plates and fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton-X 100/0.1% sodium citrate at 4°C. The cells were then washed extensively and terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling assay was performed using the *In Situ* Cell Death Detection kit/TMR Red (Roche Applied Science). The reaction was stopped after 1 h, and the number of apoptotic cells in each well was counted under a confocal microscope.

FAS enzyme assay. The enzyme activity of FAS was assayed as described previously (28). Briefly, the cells were grown in 12-well plates with or without cyclophosphamide and cerulenin. After 24 h, the cells were collected and resuspended with 0.25 mol/L Sucrose buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L Tris-HCl, and 1 mmol/L DTT), and the cells were then homogenized by a type A Dounce homogenizer. FAS activity was measured spectrophotometrically by monitoring oxidation of NADPH (Sigma). Fifty microliters of the cell extract were added to a 500 μL reaction mixture containing 0.1 mol/L K_2HPO_4 (pH 7.0), 0.3 mmol/L NADPH, and 0.05 mmol/L Acetyl-CoA, and the absorbance at 340 nm was monitored for 3 min to measure background of NADPH oxidation. Malonyl-CoA

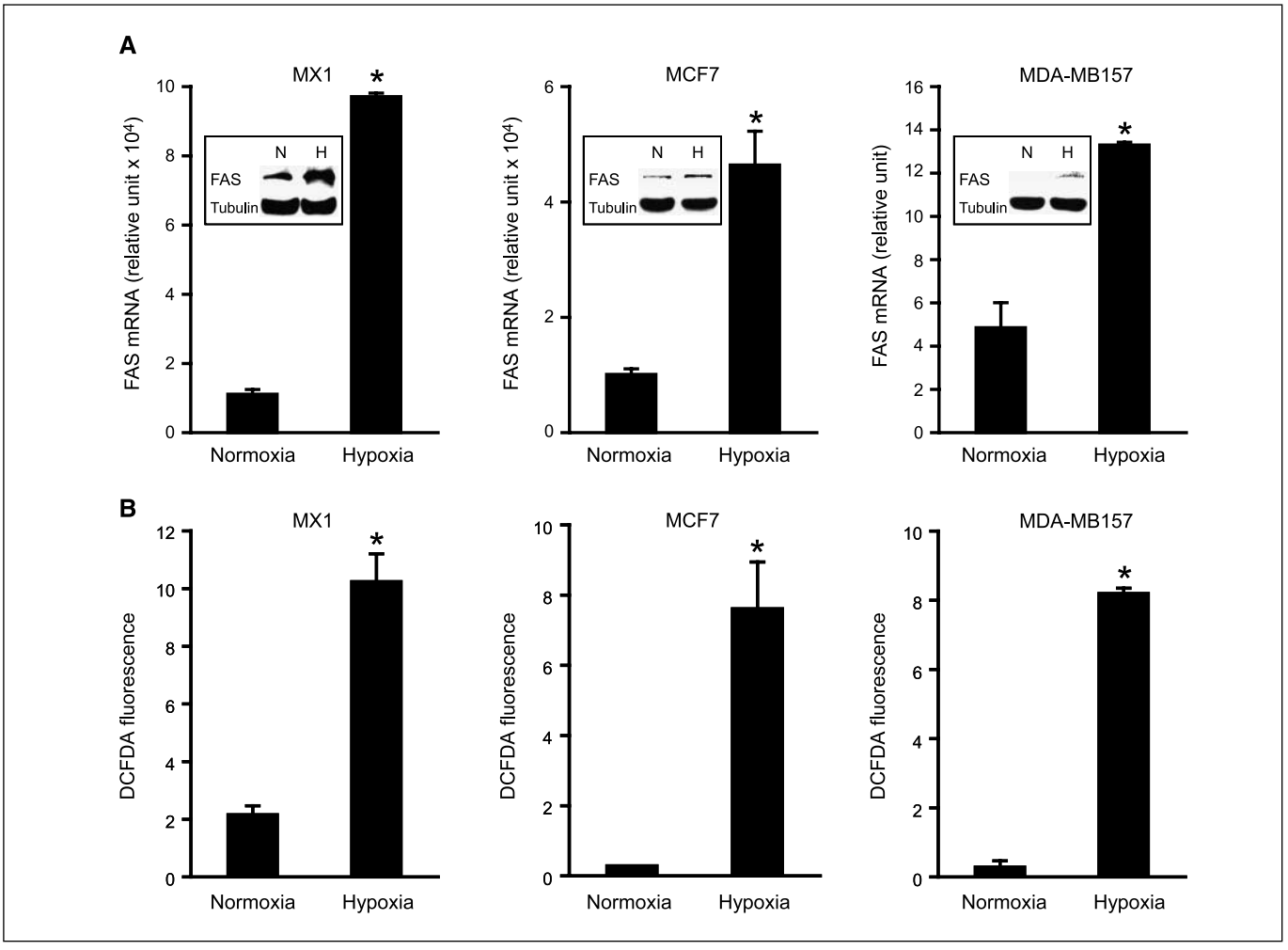


Figure 1. Hypoxia significantly augments the expression of FAS. *A,* human breast cancer cell lines, MX1, MCF7, and MDA-MB157 were cultured in three sets of 24-well plates under normoxic (*N*) or hypoxic (*H*) conditions for 48 h. One set of cells (in triplicate) was collected, and RNA was prepared. The samples were then subjected to qRT-PCR using primers for the FAS and β-actin genes. Another set of cells was collected, and the cell lysates were subjected to Western blot analysis using anti-FAS and antitubulin antibodies (*inset*). *B,* the last set of plates was used for assaying the amount of ROS using DCFDA dye. The cells were treated with the dye for 1 h followed by washing the wells with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by using a MCID software.

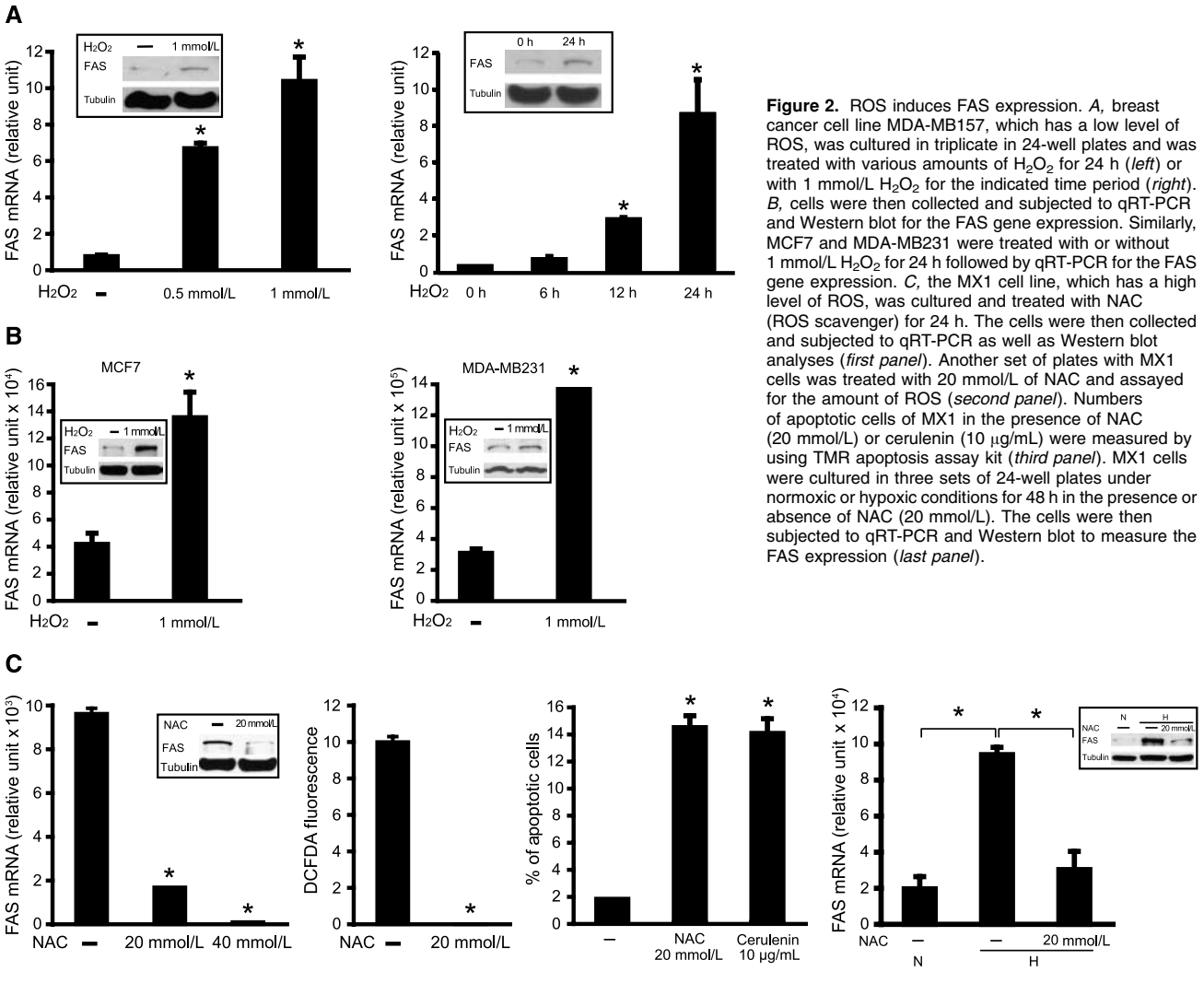


Figure 2. ROS induces FAS expression. *A,* breast cancer cell line MDA-MB157, which has a low level of ROS, was cultured in triplicate in 24-well plates and was treated with various amounts of H_2O_2 for 24 h (*left*) or with 1 mmol/L H_2O_2 for the indicated time period (*right*). *B,* cells were then collected and subjected to qRT-PCR and Western blot for the FAS gene expression. Similarly, MCF7 and MDA-MB231 were treated with or without 1 mmol/L H_2O_2 for 24 h followed by qRT-PCR for the FAS gene expression. *C,* the MX1 cell line, which has a high level of ROS, was cultured and treated with NAC (ROS scavenger) for 24 h. The cells were then collected and subjected to qRT-PCR as well as Western blot analyses (*first panel*). Another set of plates with MX1 cells was treated with 20 mmol/L of NAC and assayed for the amount of ROS (*second panel*). Numbers of apoptotic cells of MX1 in the presence of NAC (20 mmol/L) or cerulenin (10 μg/mL) were measured by using TMR apoptosis assay kit (*third panel*). MX1 cells were cultured in three sets of 24-well plates under normoxic or hypoxic conditions for 48 h in the presence or absence of NAC (20 mmol/L). The cells were then subjected to qRT-PCR and Western blot to measure the FAS expression (*last panel*).

(0.2 mmol/L) was then added to the reaction mixture, and absorbance at 340 nm was again monitored for 3 min to measure FAS activity.

Statistical analysis. For *in vitro* experiments, one way ANOVA was used to calculate the *P* values. Descriptive statistics comparing the expression of FAS, SREBP-1, and CA9 were analyzed by standard χ^2 test. For all of the statistical tests, the significance was defined as having a *P* value of <0.05. In all cases, SPSS software was used.

Results

Hypoxia induces the expression of the FAS gene via reactive oxygen species. To examine the effect of hypoxia on the expression of the FAS gene, we cultured three breast cancer cell lines, MX1, MCF7, and MDA-MB157, under normoxic or hypoxic conditions. The RNA and cell lysates were prepared from these samples, and the level of FAS expression was measured by quantitative reverse transcription-PCR (qRT-PCR) and Western blot. As shown in Fig. 1, our results indicate that the transcription of the FAS gene was significantly increased in hypoxia compared with that in normoxia (Fig. 1*A*). Protein level of FAS was also strongly increased in hypoxic condition (Fig. 1*A, inset*), although the amount of FAS induced in these three cell lines at protein level (7.1-, 4.5-, and 3.0-fold, respectively) seems to be less than that at RNA level (8.7-, 4.6-, and 2.7-fold, respectively). These apparent differences may be due to the instability of the mRNA or the FAS protein. We also examined the amount of reactive oxygen species (ROS) in these cells under normoxic and hypoxic conditions and found that hypoxia significantly augmented the generation of ROS in all these cell lines (Fig. 1*B*), which is in good agreement with previous reports (29). These results suggest that the expression of the FAS gene is positively controlled by hypoxia, which is also associated with the amount of ROS in the cell. To further corroborate our results, we tested the effect of H_2O_2 on the FAS expression in MDA-MB157, which displayed the lowest level of FAS. As shown in Fig. 2*A*, the addition of H_2O_2 in the culture medium significantly augmented the expression of FAS at both RNA and protein levels in a dose- and time-dependent manner. Other cell lines, MCF7 and MDA-MB231, also showed a similar trend and increased the FAS expression by 3- to 4-fold in response to H_2O_2 (Fig. 2*B*). On the other hand, addition of an ROS scavenger, NAC, significantly suppressed the expression of the FAS gene as well as ROS production in MX1, which showed the highest level of FAS expression among the tested cell lines (Fig. 2*C, first* and *second*

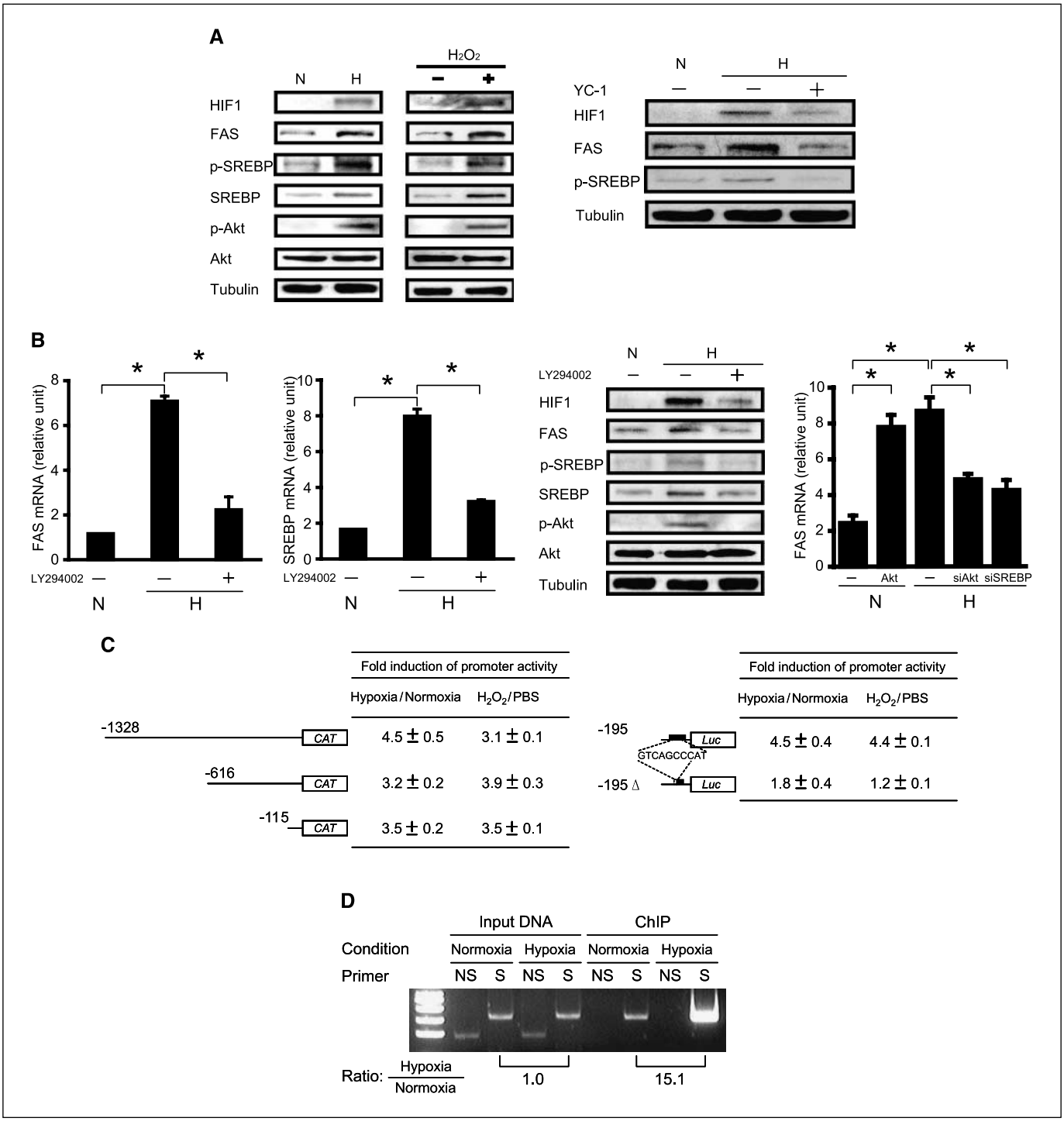
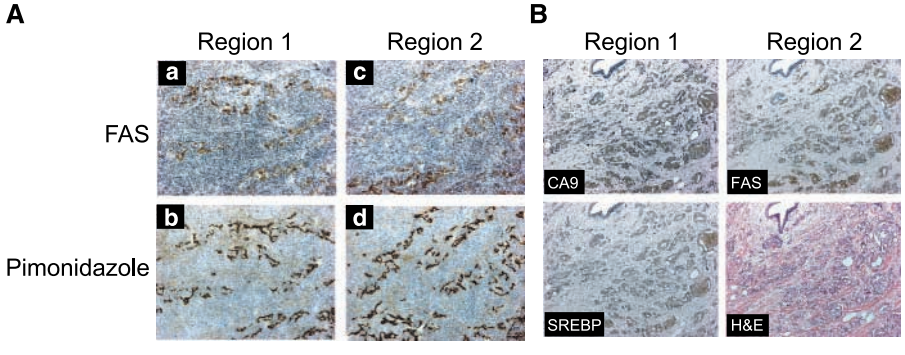


Figure 3. Hypoxia-induced FAS expression is mediated via Akt, HIF1, and SREBP-1. *A,* MCF7 cells were cultured in 24-well plates under hypoxia or normoxia conditions for 48 h and with or without H_2O_2 for 24 h. Cells were then collected and subjected to Western blot analyses using antibodies for FAS, HIF1, phospho-SREBP (*p-SREBP*), SREBP-1, phospho-Akt (*p-Akt*), total Akt (*Akt*), and Tubulin (*left*). The MCF7 cells were also cultured in the presence or absence of YC-1 (HIF1 inhibitor) under hypoxic or normoxic conditions for 48 h. Cell lysates were subjected to Western blot analyses for HIF1 and FAS expression (*right*). *B,* MCF7 cells cultured under normoxia or hypoxia with or without the treatment of LY294002 were subjected to qRT-PCR to quantify the expression of the FAS and SREBP genes (*first and second panels*). Another set of culture with the same treatment was also subjected to Western blot analysis (*third panel*). siRNA for Akt1 and SREBP-1, or the expression plasmid of active form of Akt1 were transfected to MCF7 cells. The cells were then incubated under normoxic or hypoxic conditions for 48 h. The cells were collected and subjected to qRT-PCR analysis for FAS expression (*fourth panel*). *C,* CAT reporter constructs with various lengths of the FAS promoter were transfected to MCF7, and the cells were continued to be cultured under hypoxic or normoxic conditions for 48 h and with or without H_2O_2 for 24 h. Cells were then collected, and the cell lysates were subjected to CAT assay (*left*). The luciferase reporter plasmid with 195 bases of FAS promoter with or without deletion of E-box was transfected to MCF7, and the cells were cultured under hypoxia or normoxia for 48 h and with or without H_2O_2 for 24 h. Cells were then collected and assayed for luciferase activities (*right*). *D,* for ChIP assay, MCF7 cells were cultured under normoxia or hypoxia for 24 h. The cells were lysed and the lysate was pulled down with anti–SREBP-1 antibody. The DNA was then subjected to quantitative PCR using nonspecific (*NS*) or SREBP binding site–specific primers (*S*). The ratio of the DNA was calculated based on cyclic threshold value for each reaction.

panels). Because inhibition of the FAS expression has been known to cause apoptosis (13), we also examined the effect of NAC on cell death. As shown in Fig. 2*C* (*third panel*), the treatment of the cell with NAC significantly induced apoptosis to the similar level as it was when treated with a specific inhibitor of FAS, cerulenin. To further confirm our results, we tested the effect of NAC on the FAS up-regulation under hypoxia and found that NAC indeed significantly blocked the up-regulation of FAS (Fig. 2*C, fourth panel*). Collectively, these results suggest that the expression of the FAS gene is up-regulated by hypoxia through the generation of ROS.

Hypoxia up-regulates the FAS gene expression through SREBP-1. To understand the mechanism of the hypoxia-induced expression of the FAS gene, we first examined the status of HIF1, SREBP, and Akt under normoxic and hypoxic conditions. HIF1 has been known as a key transcriptional regulator induced by hypoxia (30). SREBP is the major transcription factor of the FAS gene and has been known to be up-regulated under hypoxia (31). In fission yeast, SREBP was indeed found to function as an oxygen sensor (32). Akt is a key signal molecule for cell survival, and apoptosis and has been shown to be up-regulated under hypoxia (33). As shown in Fig. 3*A* (*left*), our results of Western blot analysis indicate that expressions of FAS, HIF1, SREBP-1, and phospho-SREBP (T426) were indeed up-regulated under hypoxia as well as in the presence of H_2O_2 in MCF7 cells. We also found that Akt was strongly phosphorylated at Ser^{435} in the same set of samples treated with hypoxia or H_2O_2, although the amount of total Akt was somewhat decreased, suggesting that PI3K/Akt pathway and SREBP-1 are involved in the activation of FAS by hypoxia and ROS (Fig. 3*A, left*). Because HIF1 was also up-regulated by hypoxia and H_2O_2, we next examined whether HIF1 was involved in the activation of FAS by adding a HIF1 inhibitor, YC-1, in the cultured cells under hypoxic condition. As shown in Fig. 3*A* (*right*), the hypoxic condition strongly up-regulated HIF1, and this up-regulation was blocked by YC-1. Interestingly, the YC-1 treatment also blocked the expression of FAS as well as phospho-SREBP-1, suggesting that HIF1 is also involved in the up-regulation of FAS and SREBP-1. The results of qRT-PCR analysis also indicate that FAS and SREBP-1 were significantly increased by the treatment of hypoxia (Fig. 3*B, first* and *second panels*). Furthermore, the results of both of our qRT-PCR and Western blot analyses indicate that the up-regulation of FAS, p-Akt, HIF1, and SREBP-1, as well as p-SREBP, were blocked by LY294002 (Fig. 3*B, first, second*, and *third panels*), suggesting that the induction of the FAS expression by hypoxia is mediated through activation of Akt followed by up-regulation of HIF1 and SREBP-1. To further verify our results, we tested the effect of siRNA specific to SREBP-1 and Akt as well as the effect of ectopic expression of an activated form of Akt on the FAS expression. We found that ectopic expression of Akt significantly augmented the FAS expression under normoxia, whereas both siRNA significantly blocked the up-regulation of FAS under the hypoxic condition (Fig. 3*B, fourth panel*). Therefore, both Akt and SREBP-1 coordinately regulate the up-regulation of hypoxia-induced FAS expression.

To identify the exact hypoxia-responding sequence on the FAS gene promoter, we generated a series of CAT reporter plasmids containing up to −1,328, −616, and −115 base of the FAS promoter, and CAT reporter activities were measured under normoxic or hypoxic conditions as well as in the presence or absence of H_2O_2. As shown in Fig. 3*C* (*left*), both hypoxia and H_2O_2 significantly increased the FAS promoter activity even when the promoter sequence was deleted to −115 bases. Because this region includes the SREBP binding/E-box sequence, to assess the functional significance of these sequences, we generated luciferase reporter plasmids with or without the SREBP binding sequence and tested their responsiveness to hypoxia and H_2O_2. The results of the reporter assay indicate that deletion of the SREBP binding/E-box sequence significantly reduced the responsiveness of the FAS promoter to hypoxia and H_2O_2 (Fig. 3*C, right*). Therefore, these results suggest that hypoxia induced the FAS gene by activating Akt followed by induction of SREBP-1, which then binds to the SREBP binding site of the FAS promoter. To examine further whether SREBP-1 indeed binds to the SREBP binding site under hypoxia, we performed ChIP assay by precipitating SREBP-chromatin complex using anti–SREBP-1



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		CA9		
		Positive	Negative	*P* value *
FAS	Positive	21	9	*P* = 0.0038
	Negative	8	20	
SREBP	Positive	22	10	*P* = 0.0037
	Negative	7	19	

Figure 4. FAS and SREBP-1 express in hypoxic regions of tumor *in vivo*. *A,* MDA-MB231 cells were transplanted into mammary fat pad of nude mice. The tumors were grown for 3 wk, and mice were injected with pimonidazole through i.p. After 2 h, tumors were excised and sliced on slides. These slides were then analyzed by immunohistochemistry using antibodies for FAS and pimonidazole. Photos are shown for two representative regions (*a–b* and *c–d*). *B,* to examine the relationship of FAS and hypoxia in tumor, human breast tumor samples from 29 patients were sectioned and subjected to immunohistochemical analysis using antibodies for FAS, SREBP-1, and CA9. Representative photos for each antibody staining with consecutively sectioned slides are shown. *C,* for each slide, fields of high and low expression of CA9 were randomly chosen and divided into two groups (CA9-positive and CA9-negative). Using consecutive slides of the identical samples, these regions were then analyzed by immunohistochemistry using anti-FAS and anti–SREBP-1 antibodies. Each sample was then further divided according to positive and negative expression of FAS and SREBP-1. To evaluate the significance, the expression of FAS and SREBP-1 in relation to CA9, χ^2 test was performed.

antibody followed by PCR, using the primers specific to the region of the SREBP binding site. Our result clearly indicates that the amount of SREBP-1 binding to this region was strongly augmented under hypoxia compared with that in normoxic condition (Fig. 3*D*).

The level of FAS expression correlates with hypoxia *in vivo*. To validate our *in vitro* results of the hypoxia-induced FAS expression, we examined the relationship between the expression level of FAS and hypoxic regions in an animal xenograft model. We first transplanted human breast cell lines, MDA-MB231, into nude mice and grew the tumor for 3 weeks. We then injected pimonidazole, which reacts with hypoxic cells, to the mice through i.p. After 2 h, tumors were excised and stained with antibodies for FAS and pimonidazole. We found that FAS expression colocalized with the area reactive to anti-pimonidazole, suggesting that hypoxic areas strongly expressed FAS in these tumors (Fig. 4*A*). To further validate these results in a clinical setting, we performed immunohistochemical analysis for clinical samples from 29 breast cancer patients using antibodies for FAS, SREBP-1, and CA9, which is a hypoxia marker (34). We first stained the samples with anti-CA9 and randomly chose positive and negative fields for each specimen. These samples were then stained with antibodies for FAS and SREBP-1. We then inspected the staining intensity of FAS and SREBP-1 in these CA9-positive and CA9-negative regions. As shown in Fig. 4*B* and *C*, of 29 CA9-positive regions, 21 were FAS positive (72%) and 8 were FAS negative. On the other hand, 20 of 29 CA9-negative samples (69%) were also FAS negative ($P = 0.0038$). CA9-positive regions were also significantly correlated with SREBP-1 expression ($P = 0.0037$). These results indicate that FAS was expressed preferentially in the region of hypoxia in breast cancer, which is consistent with our *in vitro* data. Taken together, our results of *in vitro* and *in vivo* experiments strongly suggest that the expression of the FAS gene is significantly induced by hypoxia, and that this induction is mediated by the generation of ROS followed by the activation of Akt and SREBP-1.

Inhibition of FAS overcomes hypoxia-induced chemoresistance. Development of resistance to chemotherapeutic drugs is a major clinical problem for the treatment of cancer patients. Rapidly growing tumors are often under hypoxic conditions, and hypoxia is known to induce chemoresistance (33, 35, 36). Because our results suggest that FAS is induced by hypoxia and that the high level of FAS protects tumor cells from apoptosis, we sought a possibility that inhibition of FAS expression by low concentration of a FAS inhibitor overcomes the hypoxia-induced chemoresistance. We first examined the effect of cyclophosphamide, a chemotherapeutic drug commonly used for the treatment of breast cancer, on MCF7 cells under hypoxic or normoxic condition. As shown in Fig. 5*A*, cyclophosphamide induced apoptosis in MCF7 in a dose-dependent manner under normoxic condition. However, when the cells were treated with cyclophosphamide under hypoxic condition, cells became significantly resistant to cyclophosphamide, indicating that hypoxia induced chemoresistance. We then treated MCF7 cell with a combination of a FAS inhibitor (cerulenin), a PI3K inhibitor (LY294002), and cyclophosphamide under hypoxic or normoxic condition followed by apoptosis assay. We found that a combination of these drugs synergistically enhanced the degree of apoptosis under normoxic condition (Fig. 5*B*). Importantly, the treatment of the cells with the combination of cyclophosphamide and cerulenin or LY294002 under hypoxia condition blocked hypoxia-induced resistance to cyclophosphamide. These results suggest that a combination of cerulenin and other chemotherapeutic drugs such as LY294002 synergistically induces tumor cell death, and that hypoxia-induced chemoresistance is partially blocked by suppression of the FAS expression or the Akt pathway. We also examined the enzymatic activity of FAS and found that the FAS activity was indeed significantly higher under hypoxic condition compared with that under normoxia even in the presence of cyclophosphamide and cerulenin (3.8 ± 1.1 versus 1.1 ± 0.2, respectively), and that the activity was inversely correlated with the degree of apoptosis. Although currently available FAS inhibitors are relatively toxic, using these drugs at a low concentration with a combination of other drugs may be a rational strategy for the treatment of chemoresistant tumors.

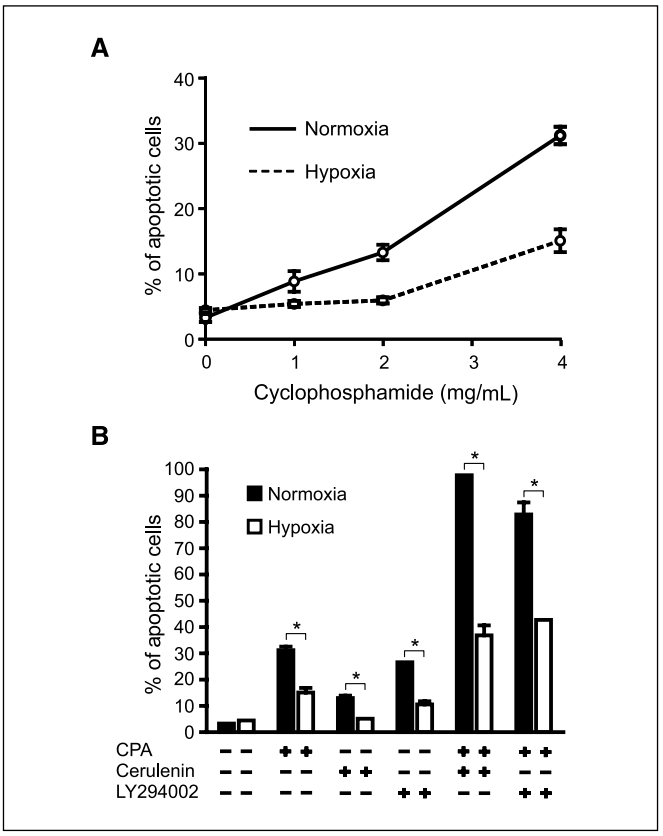

Figure 5. Inhibition of FAS overcomes hypoxia-induced chemoresistance. *A,* MCF7 cells were treated with various amounts of cyclophosphamide under normoxic (*solid line*) or hypoxic (*dotted line*) conditions for 48 h. Cells were then subjected to apoptosis assay using the TMR apoptosis assay kit. *B,* MCF7 cells were treated with suboptimum concentrations of cerulenin (10 μg/mL), LY294002 (20 μmol/L), and cyclophosphamide (4 mg/mL) either alone or in combination under hypoxia or normoxia for 48 h. Samples were then subjected to apoptosis assay as described in *A. CPA,* cyclophosphamide.

Discussion

Although the *de novo* pathway of fatty acid synthesis is quite active during embryogenesis, normal adult cells acquire fatty acids mainly from dietary source and rarely use the *de novo* pathway because nutritional fatty acid strongly suppresses the expression of the genes involved in fatty acid synthesis (3, 4). However, cancer cells are no longer sensitive to this nutritional signal and prefer to use the *de novo* pathway. In fact, linoleic and arachidonic acid, potent suppressors of the FAS gene of normal hepatic and adipocytic cells, have been shown to have no significant inhibitory effect on the expression of the FAS gene in breast cancer cells (37). Therefore, what triggers the reactivation of the FAS gene in cancer

cells and whether they use the same signal pathway as the normal cells are critical questions to understand the role of FAS in tumorigenesis. When primary tumor grows >1 mm in size, it can no longer obtain oxygen and nutrients by diffusion and requires to promote angiogenesis by inducing proangiogenic genes as a survival strategy (36). Therefore, tumor cells at an early stage are usually under hypoxic condition and at a risk of apoptosis. The reactivation of the FAS gene has been observed at a relatively early stage in various types of cancer, and these results suggest that the FAS gene is up-regulated by a common factor of cancer microenvironment such as hypoxia. In this report, we have shown that the FAS gene in cancer cell is indeed significantly up-regulated by hypoxia, and that this up-regulation is due to the activation of the Akt and HIF1 followed by up-regulation of SREBP-1.

Due to the high rate of proliferation and oxygen consumption, tumors are often under hypoxic condition, which is a hallmark of cancer. The hypoxic microenvironment is normally proapoptotic; however, tumor cells adapt themselves by inducing various enzymes to circumvent the problem. This induction is mediated by an activation of the known hypoxia-sensing pathways such as HIF1 and PI3K/Akt (38–40). In this context, it should be noted that Akt has been shown to stabilize HIF1 in both breast and prostate cancer cells (41, 42). Beitner-Johnson et al. (43) also showed that hypoxia dramatically increased phospho-Akt (Ser^{473}) in PC3 cells, and this activation of Akt was completely abolished by wortmannin, a PI3K inhibitor. It is worth noting that Akt was also found to be up-regulated by H_2O_2 (44). Consistent with these results, we have shown that hypoxia and H_2O_2 indeed induced activation of Akt (Ser^{473}) and HIF1, and that this activation was accompanied by the up-regulation of SREBP-1, a major factor involved in the regulation of the FAS gene. In cancer cells, it has been shown that PI3K/Akt signaling significantly augmented the expression of SREBP-1 in response to oncogenic signaling, including overexpression of various growth factors (11). Furthermore, we have previously shown that the tumor suppressor, PTEN, which inhibits Akt by dephosphorylation, significantly suppressed the expression of the FAS gene (22). Therefore, the activation of the Akt pathway followed by induction of SREBP-1 is considered to be one of the major pathways of reactivation of the FAS gene in cancer cell, and this reactivation is triggered at least by the hypoxic condition of tumor microenvironment. This notion is also strongly supported by our results of immunohistochemical analysis on clinical samples where FAS expression was significantly colocalized with the CA9-stained hypoxic area. It is known that Akt is quickly phosphorylated under hypoxic condition and that this activation of Akt results in up-regulation of HIF1 (45–47). Our results indeed showed that LY294002 inhibited hypoxia-induced HIF1 as well as the expression of FAS and p-SERBP-1 (Fig. 3*B*). Our results also indicate that HIF1 inhibitor, YC-1, strongly blocked phosphorylation of SREBP-1 (Fig. 3*A*), which is in good agreement with the recent finding by Li et al. (48) that HIF1 plays a key role in activation of SREBP-1 *in vivo*. Therefore, the hypoxia-induced FAS expression is considered to be mediated via phosphorylation of Akt followed by activation of HIF1 and SREBP-1.

Hypoxia generally induces apoptosis in normal cells partly due to malfunction of the respiratory system in mitochondria, which requires oxygen for ATP production (49). However, cancer cells have an unusual tolerance to hypoxic condition because they use the glycolysis pathway to generate ATP even under normoxic condition, which has been known as the Warburg effect (50). On the other hand, hypoxia was shown to cause an increase of NADH/NADPH ratio in a cell due to increased flux of glycolysis, and this change of redox balance induces inactivation of PTEN followed by activation of Akt (21). Therefore, up-regulation of FAS may be partly due to increased glycolysis and the following Akt activation. It is likely that the increased activity of FAS enhances lipogenesis, which consumes more NADPH and rebalances redox so that cells can compensate for the shortfall of oxygen.

As we and others previously reported, inhibition of the function or expression of FAS results in apoptosis of tumor cells (7–11). This cell death is considered to be caused by the suppression of CPT1 followed by accumulation of ceramide, which in turn activates proapoptotic genes such as BNIP3 (13). It should be noted that BNIP3 was found to be one of proapoptotic genes induced by hypoxia, and that specific blocking of the FAS expression by siRNA significantly increased the expression of BNIP3 followed by apoptosis (13, 51). In fact, we have shown that the expressions of FAS and BNIP3 are indeed inversely correlated in breast cancer patients (13). Therefore, FAS may act as an "antiapoptotic" gene under hypoxia. This notion is consistent with the previous observations of immunohistochemical analysis on human tumor samples where overexpression of FAS was found to be a relatively early event (7–11). We also reported that the expression of FAS was inversely related to that of PTEN in human breast tumor specimens, and the expression of higher FAS and lower PTEN is correlated to poor survival of patients, suggesting that the PTEN inactivation followed by Akt activation induced the FAS expression (22). Although the direct involvement of FAS in the initial step of tumorigenesis is yet to be determined, overexpression of FAS in tumors seems to be a survival strategy of the cancer cells to block apoptosis caused by hypoxic condition.

Because inhibition of FAS causes tumor cell apoptosis, FAS is considered to be a promising target for cancer therapy. The pharmacologic inhibitors of FAS such as cerulenin [(2*R*, 3*S*)-2,3-epoxy-4-oxo-7, 10-*trans,trans*-dodecadienamide], C75, and Orlistat have been shown to significantly suppress the cellular FAS level and also to induce apoptosis in a variety of human cancer cells including breast, prostate, colon, and ovarian cancer, although their specificity of action and potential side effects remains to be of some concern for actual clinical use (7–11). On the other hand, traditional chemotherapeutic agents commonly used for breast cancer treatment such as cyclophosphamide, carboplatin, and doxorubicin often become ineffective due to chemoresistance, particularly under hypoxic condition (52). The exact mechanism of the hypoxia-induced chemoresistance has not been well understood; however, one possible mechanism is the activation of the Akt pathway and following expression of antiapoptotic genes including FAS (33). Our results of the *in vitro* experiments clearly indicate that a FAS inhibitor, cerulenin, indeed partially overcame the hypoxia-induced chemoresistance of cyclophosphamide. Although the existing FAS inhibitors are still somewhat toxic, a use of lower concentration of these drugs in combination with the current chemotherapeutic drugs may enhance the therapeutic effect by reducing the hypoxia-induced chemoresistance.

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RhoC Promotes Metastasis via Activation of the Pyk2 Pathway in Prostate Cancer

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Abstract

***RhoC* is a member of the Ras-homologous family of genes which have been implicated in tumorigenesis and tumor progression. However, the exact role of RhoC is controversial and is yet to be clarified. We have examined the effect of RhoC on prostate tumor cells and found that RhoC had no effect on cell proliferation *in vitro* or on tumor growth in mice. However, RhoC significantly enhanced the metastatic ability of the tumor cells in these animals, suggesting that RhoC affects only the metastasis but not the growth of prostate tumor cells. The results of our immunohistochemical analyses on tumor specimens from 63 patients with prostate cancer indicate that RhoC expression had no significant correlation with Gleason grade. However, the expression of RhoC showed significant positive correlation with both lymph node and distant metastasis, and it was inversely correlated with patient survival. We also found that RhoC significantly augmented the invasion and motility of prostate tumor cells by activating matrix metalloproteinases 2 and 9 (MMP2 and MMP9) *in vitro*. The results of our antibody array analysis for signal molecules revealed that RhoC significantly activated kinases including mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt, and Pyk2. Inhibition of Pyk2 kinase blocked the RhoC-dependent activation of FAK, MAPK, and Akt, followed by the suppression of MMP2 and MMP9. Inhibitors of both MAPK and Akt also significantly blocked the activities of these MMPs. Therefore, our results indicate that RhoC promotes tumor metastasis in prostate cancer by sequential activation of Pyk2, FAK, MAPK, and Akt followed by the up-regulation of MMP2 and MMP9, which results in the stimulation of invasiveness of tumor cells.** [Cancer Res 2008;68(18):7613–20]

Introduction

The family of Ras homologous (*Rho*) genes, which plays a central role in cell proliferation and motility, has been implicated in tumorigenesis as well as metastatic progression (1). The Rho subfamily includes RhoA, RhoB, and RhoC and they share 85% amino acid sequence identity (2). Despite this similarity, each protein has different affinities with various downstream effectors and shows different subcellular localizations, suggesting that they have distinct roles in normal cellular function as well as in tumor pathogenesis (3). RhoA seems to be involved in the regulation of actomyosin contractility, and the overexpression of RhoA has been shown to promote the invasiveness of tumor cells (2, 4–6). On the other hand, RhoB plays a role in controlling cytokine trafficking as well as in apoptosis induced by DNA-damaging agents and has been suggested to act as a suppressor of tumor progression (7, 8).

Recently, RhoC has been shown to be up-regulated in various types of cancer including inflammatory breast cancer (9), hepatocellular carcinoma (10), and non–small cell lung cancer (11). However, the exact role of RhoC in tumorigenesis and tumor progression has remained controversial and needs further clarification. Pillé and colleagues previously found that blocking *RhoC* expression by short interfering RNA significantly inhibited cell proliferation of breast tumor cells *in vitro* as well as tumor growth in an animal model (12). More recently, Faried and colleagues also reported that ectopic expression of RhoC in esophageal carcinoma cells significantly enhanced the growth of tumors in nude mice. These results suggest that RhoC plays a critical role in cell proliferation and tumor growth both *in vitro* and *in vivo* (13). On the contrary, Ikoma and colleagues reported that ectopic expression of RhoC using retroviral vectors in Lewis lung carcinoma cells showed no significant difference in primary tumor growth in mice. However, the rate of lymph node metastasis was significantly enhanced in these animals (14). In agreement with these results, Hakem and colleagues recently constructed a RhoC knockout mouse and found that loss of RhoC does not affect tumorigenesis but significantly decreased metastasis in this mouse, suggesting that RhoC is involved only in metastasis but not in tumor cell proliferation (15). These apparent contradictory results by different groups may be due to the difference in the systems used or it may be due to the dependency of RhoC on cellular context. Therefore, it is critical to take a more systematic approach of testing the gene both *in vitro* and *in vivo* and to validate the outcome results in a clinical setting for each organ or tissue type in order to further clarify the role of RhoC in tumor progression. In this study, we found that RhoC promotes tumor metastasis but not tumor growth by sequential activation of Pyk2, focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and Akt followed by up-regulation of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in prostate tumor cells, and that the expression of RhoC serves as a marker to predict metastatic status and survival of patients with prostate cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture and reagents. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection, and human prostate cancer cell line PC3MM was kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PC3MM/tet cell line was previously established as a derivative of PC3MM and contains the tetracycline-inducible suppressor. Rat prostate cancer cell line AT2.1 was a gift from Dr. C.W. Rinker-Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and 250 nmol/L of dexamethasone at 37°C in a 5% CO_2 atmosphere. The phosphoinositide-3-kinase (PI3K)/Akt inhibitor (Ly294002) and the MAPK inhibitor (PD98059) were purchased from Sigma Co. and Calbiochem, respectively. FAK inhibitor (TAE226) was previously described and kindly provided by Dr. Honda (Novartis Pharma AG, Basel, Switzerland; ref. 16).

Construction of expression vectors. To generate a RhoC expression vector, cDNA of the RhoC gene was isolated by PCR amplification from a human cDNA library using a forward primer containing a Flag-tagged Kozak sequence and *Eco*RI linker and a reverse primer including a *Xho*I linker. The PCR product was then cloned into the mammalian expression vector pcDNA3 (Invitrogen). To construct a tetracycline-inducible RhoC expression plasmid, the fragment of the *RhoC* gene in pcDNA3 was subcloned into pcDNA5/TO (Invitrogen) at the *Bam*HI/*Xho*I site. The RhoC expression plasmids or the vector alone were transfected into the AT2.1, PC3MM, and PC3MM/tet cells using LipofectAMINE (Invitrogen). To establish stable clones, transfected cells were treated with G418 or hygromycin, and drug-resistant colonies were selected followed by testing RhoC expression by Western blot.

Short hairpin RNA. Five individual short hairpin RNAs (shRNA) against the *Pyk2* gene were purchased from Open Biosystems. shRNA with a scrambled sequence was purchased from Addgene and used as a negative control. The shRNAs were transfected into the prostate cancer cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and the culture was further incubated for 48 h before harvesting the cells for assays.

Western blot analysis. Cells were collected and dissolved in loading dye solution (125 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 10% β2-mercaptoethanol, and 0.04% bromophenol blue), boiled for 5 min and subjected to 8% to 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were then treated with antibodies against anti-Flag (Sigma-Aldrich), anti–β-tubulin (Upstate Biotechnology), anti–phospho-Pyk2 ($Tyr^{579/580}$; Sigma-Aldrich), anti-Pyk2 (Cell Signaling Technology), anti–phospho-Akt (Ser^{473}; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti–phospho-FAK (Tyr^{397}; Sigma-Aldrich), anti-FAK (Cell Signaling Technology), or anti–phospho-MAPK (Thr^{183}; Sigma-Aldrich) or anti-MAPK (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies and visualized by the enhanced chemiluminescence plus system (Amersham Life Sciences).

Cell growth assay. Cell lines expressing or not expressing the *RhoC* gene were cultured in the RPMI 1650 medium. At each time point, cells were trypsinized, serially diluted, and re-plated in Petri dishes. The resultant colonies were stained with crystal violet and the number of colonies was visually counted. For thymidine uptake assays, cells were treated with or without tetracycline for 24 h and ^{3}H-thymidine was added to the culture. After 3 and 12 h, cells were collected and acid-insoluble radioactivities were measured by scintillation counter.

Spontaneous metastasis assay. Rat prostate tumor cells AT2.1 (0.5×10^6 cells in 0.2 mL of PBS) were injected s.c. in the dorsal flank of 5-week-old severe combined immunodeficiency (SCID) mice (Harlan Sprague-Dawley). Mice were monitored daily and the tumor volume was measured as an index of the growth rate using the equation: volume = (width + length) / 2 × width × length × 0.5236. The doubling time of tumors during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Immunohistochemical analysis. Formaldehyde-fixed and paraffin embedded tissue specimens from 63 patients with prostate cancer were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four-micron-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L of sodium citrate buffer (pH 9.0) at 80°C for 30 min. The slides were incubated overnight at 4°C with anti-RhoC antibody (Santa Cruz Biotechnology) or anti–phospho-Akt (Ser^{473}; Cell Signaling Technology). The sections were then incubated with the horseradish peroxidase–conjugated anti-goat secondary antibody, and 3,3′-diaminobenzidine substrate chromogen solution (Envision Plus kit; DAKO, Corp.) was applied followed by counterstaining with hematoxylin. Immunohistochemical staining conditions with other antibodies (NDRG1, AR, and PTEN) were described previously (17). Results of the immunohistochemistry for RhoC were judged by two independent persons (M. Iiizumi and K. Watabe) based on the intensity of staining combined with the percentage of cells with positive staining.

***In vitro* motility and invasion assay.** For the motility assay, 1×10^5 cells were added to the cell culture inserts with microporous membrane without any extracellular matrix coating (Becton Dickinson) and RPMI medium containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37°C, and the upper chamber was removed. The cells on the bottom of the upper chambers were stained with tetrazolium dye, and the number of cells was counted under a microscope. For the *in vitro* invasion assay, the working method was similar to that described above, except that the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson).

Wound-healing migration assay. Cells were seeded in a 10-cm dish and cultured to confluency. The cell monolayer was then scraped in the form of a cross with a plastic pipette tip. Three "wounded" areas were marked for orientation and photographed by a phase contrast microscopy before and after 24 h of incubation.

Real-time reverse transcription-PCR. Forty-eight hours after transfection of appropriate plasmid DNA to the cells or 48 h after induction by tetracycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Biosystems). The cDNA was then amplified with a pair of forward and reverse primers for *RhoC* (5′-TAAGAAGGACCTGAGGCAAG and 5′-ATCTCAGAGAATGGGACAGC), *MMP2* (5′-TGATGGTGTCTGCTGGAAAG and GACACGTGAAAAGTGCCTTG), *MMP9* (5′-GGAGACCTGAGAACCAATCTC and 5′-TCCAATAGGTGATGTTGTGGT), human *β-actin* (5′-TGAGACCTTCAACACCCCAGCCATG and 5′-GTAGATGGGCACAGTGTGGGTG), *Pyk2* (5′-GCTAGACGGCAGATGAAAGT and 5′-AAGCAGACCTTGAGGATACG). PCRs were done using the Dynamo SYBRGreen qPCR kit (New England Biolabs) and DNA Engine Opticon2 System (MJ Research). The thermal cycling conditions were composed of an initial denaturation step at 95°C for 5 min followed by 30 cycles of PCR using the following profile: 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s.

Gelatin zymograph assay. For zymography assay, cells (2.5×10^5) were seeded in 12-well plates and incubated for 48 h. Supernatants were collected and mixed with sample buffer followed by electrophoresis on a 10% SDS-polyacrylamide gel containing 5 mg/mL of gelatin. The gel was washed with 2.5% Triton X solution for 2 h and further incubated in the reaction buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 1% Triton X-100) for an additional 18 h at room temperature. The gel was then stained with 0.5% Coomassie blue for 9 h and subsequently immersed with destaining buffer (30% methanol, 10% acetic acid) for 12 h. The image was photographed and the intensity of each band was digitally quantified.

Antibody microarray. Antibody microarray was performed using a Panorama Antibody Microarray-Cell Signaling kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 1.5×10^7 cells were seeded in T-75 flasks and incubated for 48 h in the medium with or without tetracycline. Cells were collected and protein samples were prepared according to the manufacturer's protocol. These protein samples were labeled with Cy3 or Cy5 (Amersham Biosciences, UK) and subjected to antibody microarray (Sigma-Aldrich) analysis. The array slides were

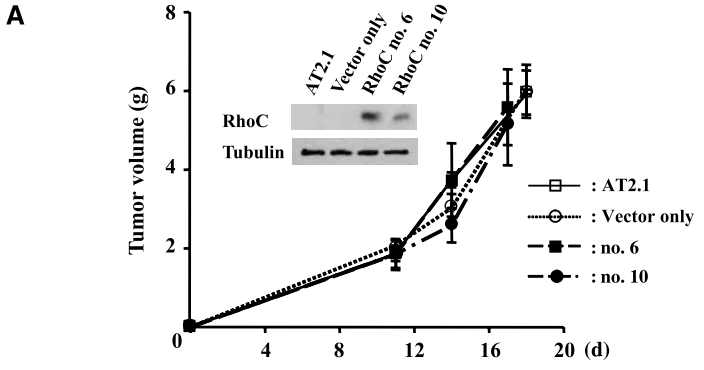
scanned by GenePix Personal 4100A scanner (Molecular Devices) and the data was analyzed by GenePix Pro 5.0 (Molecular Devices).

Statistical analysis. For *in vitro* experiments and animal studies, *t* test or one-way ANOVA was used to calculate the *P* values. The association between RhoC and other clinical markers was calculated by χ^2 test. The Kaplan-Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log-rank test. Univariate and multivariate analyses for the prognostic value of RhoC was performed by the Cox proportional hazard-regression model. For all of the statistical tests, the significance was defined as $P < 0.05$. SPSS software was used in all cases.

Results

RhoC promotes tumor metastasis, but not cell growth. To understand the role of RhoC in prostate cancer, we first established permanent cell lines expressing RhoC using the rat prostate carcinoma cell line, AT2.1, which has a poor metastatic potential (18). These cell lines expressing RhoC (clone no. 6 and no. 10) and a clone containing only the vector as well as the parental cell line, AT2.1, were individually injected s.c. into SCID mice. The mice were monitored for the formation and the growth rate of tumors and then sacrificed 3 weeks after the inoculation of the cells. As shown in Fig. 1*A*, all of the clones and the parental cells formed primary tumors in the animals with similar growth rates during the 3-week period, suggesting that RhoC does not have an effect on tumorigenesis or tumor growth. On the other hand, the clones stably expressing RhoC showed a significantly higher incidence of lung metastases compared with the parental cell line and the vector-only clones (Fig. 1*B*). These results strongly suggest that RhoC can promote the metastatic process of prostate cancer cells without affecting tumorigenicity *in vivo*. We also examined the effect of RhoC on the growth of these cells *in vitro*. The results of a colorimetric assay after 72 h indicate that there was no significant difference in the growth rate between the cells with and without RhoC (Supplementary Fig. S1*A*). We then examined the rate of DNA synthesis of the cells with and without the expression of RhoC and found that there was no significant difference between these cells (Supplementary Fig. S1*B*). Furthermore, we established a human prostate cell line, PC3MM/tet/RhoC, which contains the tetracycline-inducible *RhoC* gene, as well as PC3 cell lines that did or did not ectopically express RhoC. We then examined the rate of cell growth and DNA synthesis of these cells. Again, we found that RhoC did not affect the rate of proliferation of the cells (Supplementary Fig. S1*A* and *B*), which further supports our notion that RhoC has no apparent role in the growth of prostate cancer cells, although it significantly promotes tumor metastasis.

RhoC expression is significantly increased with the advancement of human prostate cancer. To further corroborate our results in a clinical setting, we examined the status of RhoC expression and its relationship with different clinicopathologic factors in prostate cancer by immunohistochemical analysis of 63 prostate tumor specimens. They were randomly selected from surgical pathology archives dating from 1988 to 2001. As shown in Fig. 2*A* and *B*, the expression of RhoC was found to be strongly elevated in high-grade tumors, particularly in specimens from patients with metastatic disease, compared with normal prostatic tissue or low-grade tumors. The results of our statistical analyses indicate that RhoC is strongly expressed in tumors with higher Gleason grade, although the correlations are not statistically significant (Fig. 2*B*). Importantly, the RhoC expression showed significant positive correlation with the metastases status of the patients ($P = 0.028$). It was also noted that RhoC expression showed a significant inverse correlation to that of NDRG1 ($P = 0.02$), which



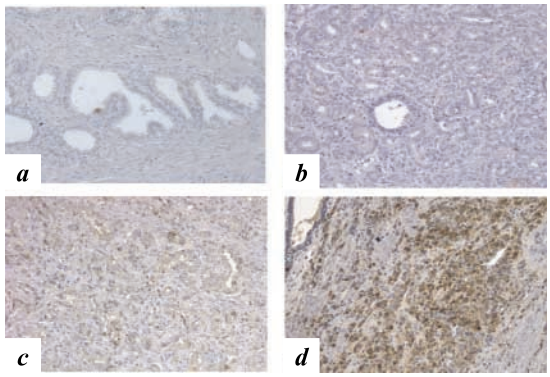
B

Cell line	RhoC*	Tumor incidence†	*In vivo* doubling time	Lung metastasis (mean ± S.E.)‡	*P*
AT2.1	–	7/7	4.1 ± 0.5	3.1 ± 1.3	
Vector only	–	4/4	3.9 ± 0.3	2.0 ± 1.1	
RhoC no. 6	+	8/8	3.9 ± 1.1	10.9 ± 3.5	0.04§
RhoC no. 10	+	5/5	3.8 ± 0.7	9.4 ± 2.4	0.03§

* RhoC expression was examined by Western blot.
† Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.
‡ Number of metastatic lesions on lungs per SCID mouse.
§ $P < 0.05$.

Figure 1. RhoC promotes tumor metastasis without affecting the primary tumor growth *in vivo*. The RhoC expression plasmid was introduced into a low-metastasis rat prostate cell line, AT2.1, and clones (no. 6 and no. 10) that constitutively express RhoC were established. As a control, the original vector was also cloned into AT2.1. These clones, as well as the parental line, were injected s.c. into SCID mice as described previously. The volume of the primary tumor for each clone at the indicated time was measured using the equation: volume = (width + length) / 2 × W × L × 0.5236 (*A*). *Inset,* results of a Western blot of RhoC expression for each clone. Mice were sacrificed 3 wk after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically (*B*). §, $P < 0.05$, statistically significant difference.

A



B

Relationship between RhoC and other clinical variables

Factor	All (63)	RhoC expression		*P*
		Positive (33)	Reduced (30)	
Age (y)				
> 71	41	20 (48.78%)	21 (51.22%)	
≤ 70	22	13 (59.09%)	9 (40.91%)	0.605
Gleason grade				
≥ 7	33	21 (63.64%)	12 (36.36%)	
< 7	30	12 (40%)	18 (60%)	0.104
Androgen receptor				
Positive	50	29 (58%)	21 (42%)	
Negative	13	4 (30.77%)	9 (69.23%)	0.150
PTEN				
Positive	39	18 (46.15%)	21 (53.85%)	
Reduced	24	15 (62.5%)	9 (37.5%)	0.316
NDRG1				
Positive	40	16 (40%)	24 (60%)	
Reduced	23	17 (73.91%)	6 (26.09%)	0.02*
Metastasis status				
Organ-confined	34	14 (41.18%)	20 (58.82%)	
Lym/bone	26	19 (73.08%)	7 (26.92%)	0.028*

Figure 2. Immunohistochemical analysis of RhoC in human prostate cancer. Immunohistochemical staining was performed on paraffin-embedded human prostate tissue sections using anti-RhoC antibody and the results were compared with other clinical variables. *A,* representative field with immunostaining for RhoC in normal prostate tissue (*a*), low-grade carcinoma (*b*), high-grade localized carcinoma (*c*), and high-grade metastatic carcinoma tissue (*d*). *B,* association of RhoC with other clinical variables was analyzed by standard χ^2 test using SPSS software. *, $P < 0.05$, statistically significant difference.

has recently been shown to be a tumor metastases suppressor in prostate cancer (19). These results suggest that the expression of RhoC is up-regulated at a relatively late stage and is directly involved in metastatic progression of prostate cancer, which is in good agreement with our *in vivo* data. Furthermore, the results of our survival analyses on 50 patients with prostate cancer over a period of 5 years indicates that patients with positive expression of RhoC had significantly worse overall survival rate than the patients with a reduced expression of the gene ($P = 0.018$, log-rank test; Fig. 3). The results of univariate Cox regression analysis revealed that the death risk of patients with increased RhoC expression was 4.8 times higher than the risk of patients with RhoC negativity. However, when we performed a multivariate analysis for RhoC, Gleason score, and metastasis, only the metastasis status gave a significant value ($P = 0.015$) and other two factors were excluded. The fact that multivariate analyses of these three factors excluded RhoC status indicates that the profiles of the RhoC expression and metastasis status of patients significantly overlaps and that each factor has enough "power" for predicting patient outcome. In fact, when we did a multivariate analysis for a combination of RhoC status and Gleason score, which is the most widely used pathologic marker for prostate cancer, RhoC status turned out to be a better predicting marker than Gleason score ($P = 0.037$ and $P = 0.237$ for RhoC and Gleason score status, respectively). Although RhoC expression did not significantly and independently predict survival compared with metastasis, increased RhoC correlates with aggressive disease which could account for increased metastatic disease.

RhoC promotes invasiveness and motility of prostate cancer cells *in vitro*. To understand how RhoC contributes to the progression of prostate cancer, we ectopically expressed the *RhoC* gene in the human prostate cancer cell line, PC3, followed by examining the invasiveness and migration of the cells *in vitro*. We found that the expression of RhoC significantly enhanced both cell

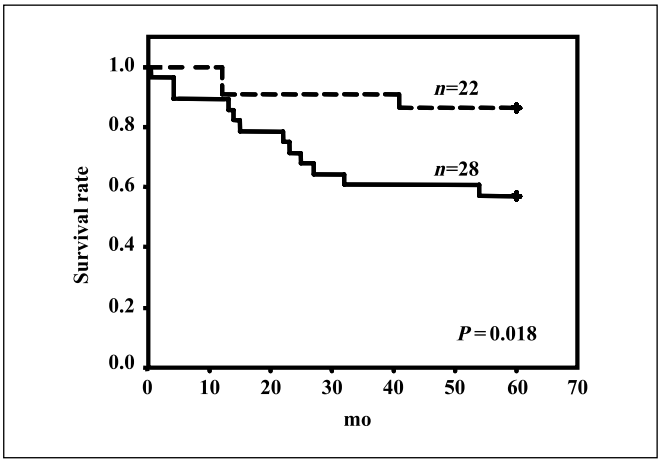


Figure 3. Prognostic value of RhoC expression. Overall survival rate over a period of 5 y was calculated in 50 patients with prostate cancer in relation to the expression of the *RhoC* genes by Kaplan-Meier method. $P = 0.018$ was determined by a log-rank test. RhoC-positive (*solid line*) patients and patients with reduced expression (*dotted line*) of RhoC.

invasiveness and migration ($P = 0.03$ and 0.004, respectively; Fig. 4*A*), which is in good agreement with the previous results of Yao and colleagues (20). The effect of RhoC on cell motility was also examined by the "wound healing" assay. As shown in Fig. 4*B*, cells with ectopically expressing RhoC showed a much higher rate of motility compared with the cells with an empty vector transfectant. These results strongly suggest that RhoC promotes metastasis by enhancing the invasiveness and/or motility of tumor cells. Because the invasive ability of tumor cells is known to often be correlated with their production of secretory proteases (21), we examined the expression of MMP2 and MMP9 in the cells that overexpressed RhoC. As shown in Fig. 4*C*, quantitative reverse transcription-PCR (qRT-PCR) analysis for the cell overexpressing RhoC significantly augmented the level of the expression of the *MMP2* and *MMP9* genes ($P = 0.049$ and 0.02, respectively). These results were further validated by gelatin zymography and Western blot analyses as shown in Fig. 4*D*. Therefore, our results indicate that the invasiveness of tumor cells induced by RhoC is, at least in part, due to the overexpression of MMP2 and MMP9.

RhoC activates MMP through the Pyk2 signal pathway. To gain further insight into the signaling pathways by which RhoC promotes the invasive phenotype, we prepared cell lysates from PC3MM/tet/RhoC with or without induction of the *RhoC* gene by tetracycline. The lysates were labeled with Cy3 and Cy5 and analyzed on an antibody microarray which contained 224 antibodies for various key molecules of cell signaling and cell cycle, and the results of ratios were rank-ordered. As shown in Fig. 5*A* (*left*), ectopic expression of RhoC significantly phosphorylated a series of protein kinases including MAPK, FAK, Akt, and Pyk2. The result of the array analysis was also confirmed by Western blot using the antibodies specific to phosphorylated proteins as well as the antibodies to the total proteins for each signal molecule (Fig. 5*A, right*; Supplementary Fig. S2*A*). These results suggest that RhoC can directly activate a cascade of signal pathways involving these key signal molecules that are closely related to cell motility and tumor progression.

Pyk2 is a tyrosine kinase and belongs to a member of the FAK subfamily which plays a critical role in cell migration and motility of various cell types (22, 23). Pyk2 is also known to be able to phosphorylate Akt (23). Therefore, we investigated the possibility

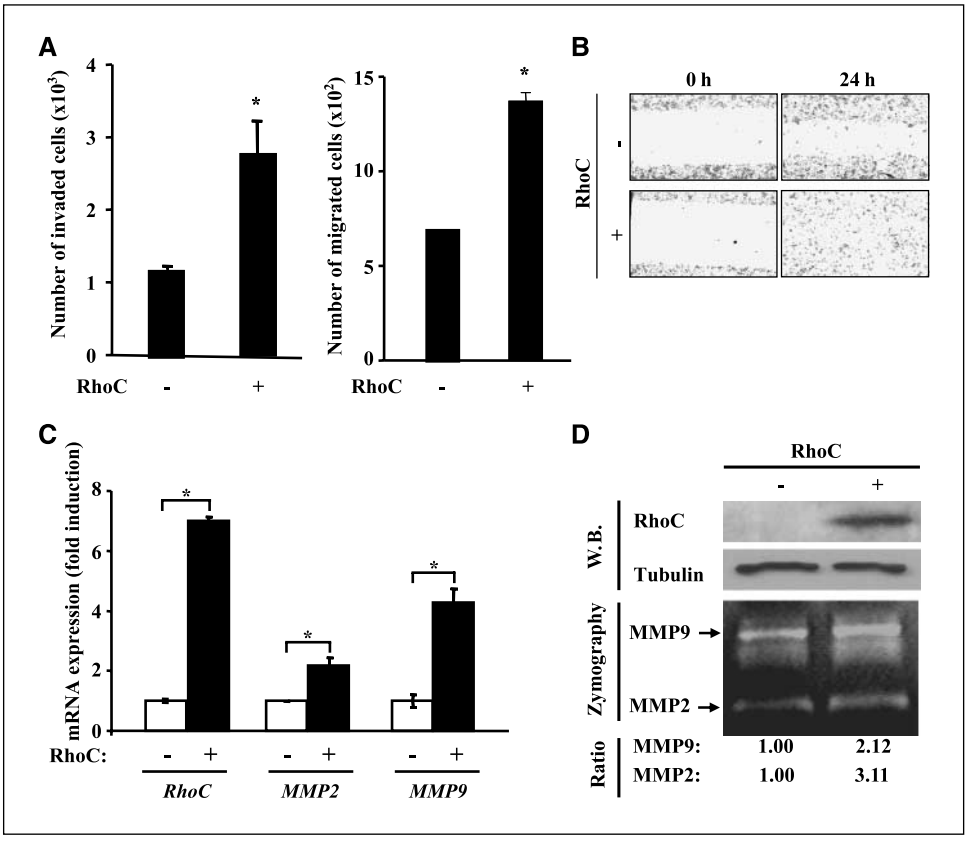
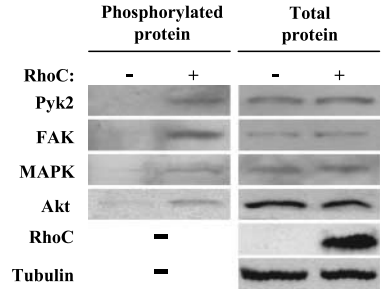


Figure 4. RhoC promotes invasiveness and motility of prostate cancer cells *in vitro. A,* the RhoC expression plasmid (pcDNA3/RhoC) or the vector alone was transfected into the PC3 cell line. After 24 h, cells were collected and subjected to invasion (*left*) and migration (*right*) assays. *, $P < 0.05$, statistically significant difference. *B,* for the motility assay, the PC3 cells stably transfected with the RhoC expression plasmid or an empty vector were cultured to confluency. The monolayer was scratched by drawing lines and photographed under a microscope. After 24 h of incubation, they were photographed again. *C,* to test the effect of RhoC on MMP2 and MMP9, PC3 cells that have been stably transfected with the RhoC expression plasmid or an empty vector were cultured in 12-well plates. Cells were then collected and their total RNA was treated with DNase. The RNA was then subjected to qRT-PCR using specific primers for the *RhoC, MMP2*, and *MMP9* genes. Results were presented as ratios of the expression level of each gene in RhoC-positive and RhoC-negative cells. *, $P < 0.05$, statistically significant difference. *D,* MMP2 and MMP9 activities in the conditioned medium from the PC3 cells with or without the RhoC expression plasmid as described in *C* were assayed by gelatin zymography. The image was photographed and the intensity of each band was digitally quantified. The expression of Flag-RhoC was confirmed by Western blot (*top*).

A

Gene Name	Phosphorylation site	Ratio (Cy5/Cy3)
MAPK	Thr^{183}	2.85
FAK	Ser^{910}	2.47
PKB/Akt	Thr^{308}	2.41
FAK	Tyr^{577}	2.38
FAK	Ser^{722}	2.35
MAPK	Thr^{183}/Tyr^{185}	2.35
FAK	Ser^{473}	2.31
PyK2	Tyr^{881}	2.26
MAPK	Tyr^{185}	2.21
PAK	Ser^{212}	2.2
PyK2	$Tyr^{579/580}$	2.2
FAK	Tyr^{397}	2.14



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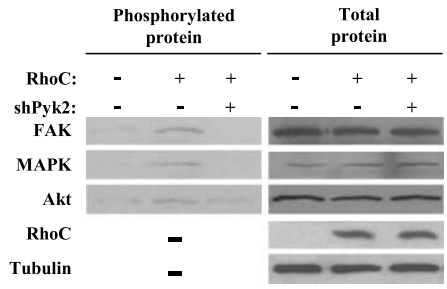

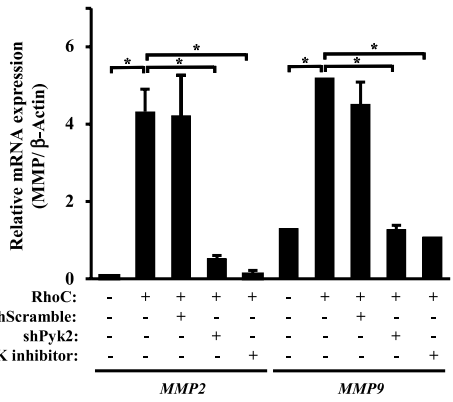

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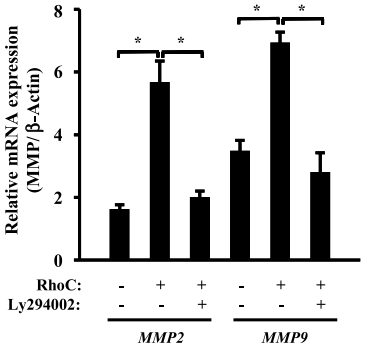

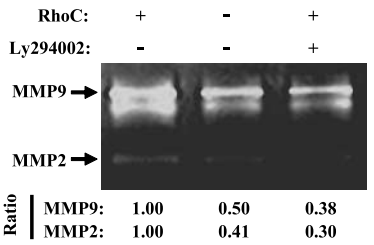

D

	RhoC expression		
All (27)	Positive (13)	Reduced (14)	*P*
P-Akt (Ser^{473})			
Positive (14)	10	4	
Reduced (13)	3	10	0.033*

E

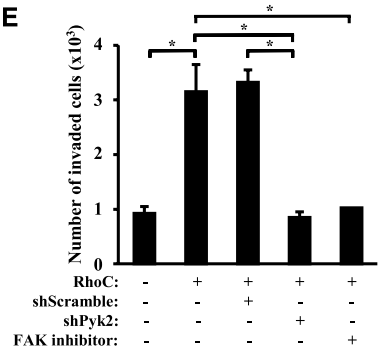

Figure 5. RhoC activates MMPs through the Pyk2/FAK pathway. *A,* for antibody array analysis, cell lysates were prepared from the PC3MM/tet cells containing the tetracycline-inducible *RhoC* gene with or without induction of RhoC. The proteins were labeled with Cy3 or Cy5 and subjected to antibody microarray (Sigma-Aldrich) analysis. The scanned data was analyzed by GenePix Pro 5.0 (Axon Instrument). The result of the antibody array data was confirmed by Western blot using phosphospecific antibodies to Pyk2, FAK, MAPK, and Akt as well as using antibodies to the total protein of each corresponding gene. *B,* PC3 cells stably transfected with the RhoC-expression plasmid or an empty vector were transfected with the expression plasmid of shRNA for *Pyk2* or a scrambled sequence. After 48 h, cells were collected and subjected to Western blot analysis using phospho-specific antibodies (*left*). To examine the effect of Pyk2 and MAPK on the MMP expression, the same set of cells were treated with or without the MAPK inhibitor, PD98059 (100 μmol/L) for 48 h. RNA was extracted from each sample (in triplicate) and subjected to qRT-PCR using specific primers for *MMP2* and *MMP9* (*right*). *C,* the effect of Akt phosphorylation on MMP expression was examined. Cells with or without expression of RhoC were treated with or without PI3K/Akt inhibitor, Ly294002 (100 nmol/L), for 48 h. The cells were then collected and RNA was extracted followed by qRT-PCR analysis for *MMP2* and *MMP9* expression (*left*). The conditioned culture mediums of the same set of samples were subjected to zymography assay for MMP2 and MMP9 (*right*). The image was photographed and the intensity of each band was digitally quantified. *D,* to examine the clinical status of RhoC and p-Akt expression, 27 samples from patients with prostate cancer were analyzed by immunohistochemistry using antibodies to RhoC and p-Akt. The result was analyzed by χ^2 test. *E,* PC3 cells with or without RhoC expression were treated with shPyk2 or the FAK-specific inhibitor, TAE226, for 48 h. The cells were then assayed for their invasiveness by using a Matrigel invasion assay as described in Materials and Methods.

that Pyk2 is an immediate effector of the RhoC signal and that it controls the downstream pathways. PC3/RhoC cells were transfected with the expression vector of shRNA targeted to *Pyk2*. After 48 h of incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies to RhoC, p-FAK, p-MAPK, and p-Akt. As shown in Fig. 5*B* (*left*) and Supplementary Fig. S2*B*, induction of RhoC strongly phosphorylated FAK, MAPK, and Akt, and this RhoC-dependent phosphorylation of these molecules was strongly blocked by the addition of shRNA to the *Pyk2* gene, suggesting that RhoC first activates Pyk2, which then phosphorylates FAK, MAPK, and Akt. We then examined whether MMP2 and MMP9 are indeed activated by Pyk2 and MAPK in a RhoC-dependent manner. RNA was prepared from PC3/RhoC cells that were cultured in the presence or absence of shRNA for *Pyk2* and the MAPK inhibitor, PD98059. RNAs were then examined for the expression of *MMP2* and *MMP9* by qRT-PCR. As shown in Fig. 5*B* (*right*) and Supplementary Fig. S2*C* (*left*), RhoC-dependent activation of both MMP2 and MMP9 was significantly abrogated in the presence of shRNA for *Pyk2* or the MAPK inhibitor, suggesting that the activation of MMP2 and MMP9 by RhoC is at least partly due to the phosphorylation of Pyk2 followed by the activation of MAPK. Because our results indicate that Akt is also phosphorylated at Ser^{473} by RhoC in a Pyk2-dependent fashion, we examined whether Akt is also involved in the activation of MMP2 and MMP9 in the RhoC signal pathway. As shown in Fig. 5*C* (*left*) and Supplementary Fig. S2*C* (*right*), we found that the RhoC-dependent induction of *MMP2* and *MMP9* was indeed significantly blocked by PI3K/Akt inhibitor, Ly294002. This result was further confirmed by gelatin zymography analysis as shown in Fig. 5*C* (*right*). To further corroborate the *in vitro* results, we examined 27 clinical specimens from patients with prostate cancer by conducting immunohistochemistry using anti-RhoC and anti–phospho-Akt (Ser^{473}) antibodies. As shown in Fig. 5*D*, we found that RhoC expression was significantly correlated with the expression of phospho-Akt in these tumor tissues. Therefore, these clinical data as well as the *in vitro* results strongly suggest that Akt is part of the downstream effectors of RhoC signals and plays an important role in RhoC-dependent activation of MMP2 and MMP9. To further validate the role of Pyk2 and FAK in the RhoC-induced signal, we treated the PC3 cells that do or do not express RhoC with shPyk2 or the FAK-specific inhibitor, TAE226, followed by measuring the invasiveness of these cells using the Matrigel invasion chamber assay. As shown in Fig. 5*E*, we found that inhibition of Pky2 and FAK indeed significantly blocked the RhoC-induced invasiveness of the prostate tumor cells, which strongly suggests the functional involvement of Pyk2 and FAK in the RhoC signaling pathway.

Discussion

RhoC has been shown to be involved in various types of tumors (9–11). However, the exact role of RhoC in tumor progression and its underlying mechanism are unclear, and the previous results from different groups have presented an apparently contradictory picture of the function of this gene (12–15). In this study, we have integrated multiple approaches, both *in vitro* and *in vivo*, to clarify the functional role of RhoC in prostate cancer progression. The results of our animal experiments clearly indicate that RhoC plays a critical role in the metastatic progression of prostate tumor but it is not essential for tumor cell growth. The results of immunohistochemical analysis of human prostate cancer specimens also indicates that RhoC expression is significantly correlated with the metastatic status of the patients but not with Gleason grade, which strongly supports our notion that RhoC is implicated mainly in the metastatic process but not in tumorigenesis. Importantly, RhoC expression is inversely correlated with patient survival, suggesting that RhoC can serve as a prognostic marker as well as a potential therapeutic target for prostate cancer.

The molecular mechanism by which RhoC promotes tumor progression is an intriguing question. We have constructed a RhoC-inducible cell line and examined its protein expression profile using an antibody array to clarify the signal pathway. The results of the array analysis revealed that Pyk2, FAK, MAPK, and Akt were all phosphorylated upon induction of the RhoC expression, and the knockdown of *Pyk2* resulted in significant reduction in phosphorylation of FAK, MAPK, and Akt, suggesting that Pyk2 is the upstream effector and plays a central role in the RhoC signal pathway. Pyk2 belongs to the subfamily of focal adhesion protein tyrosine kinases and it has been shown to be involved in cell migration, invasion, and proliferation (24–28). It was reported that in the *in vitro* model of transforming growth factor-β–induced epithelial to mesenchymal transition, Pyk2 was strongly phosphorylated at Tyr^{881} whereas during migration, Pyk2 was strongly phosphorylated at Tyr^{580} (22). It should be noted that, in our antibody array analyses, both of these sites were found to be phosphorylated (Fig. 5*A*). Pyk2 is capable of transducing signals via several known pathways, and one of the effectors is FAK which has been shown to be phosphorylated by Pyk2 at Tyr^{397}, $Tyr^{576/577}$, and Tyr^{925} (29). The results of our antibody array data also revealed that both of these sites were indeed phosphorylated upon induction of RhoC. These results suggest that RhoC activates FAK via phosphorylation of Pyk2. FAK is a focal-adhesion kinase and plays a critical role in cell migration and motility (30–32). The enhanced expression of FAK has been documented in a number of different types of human cancers (33–41). The phosphorylation of FAK is known to be linked to the activation of several downstream signals including ERK and JNK/MAPK as well as PI3K/Akt (42, 43). Furthermore, it was previously shown that the invasive ability of RhoC was significantly attenuated by a MAPK inhibitor *in vitro* (44). Notably, the results of our knockdown experiments using *Pyk2*-specific shRNA has shown that the RhoC-dependent phosphorylation of both ERK/MAPK and Akt was significantly blocked by knockdown of *Pyk2*, suggesting that MAPK and Akt are activated by RhoC via phosphorylation of Pyk2 and FAK.

We have shown that RhoC promotes metastasis by augmenting the motility and invasion of tumor cells (Figs. 4 and 5) via activation of MMP2 and MMP9, two key proteases for the invasion of tumor cells. It should be noted that the expression of both MMP2 and MMP9 was previously shown to be modulated by the activation of Akt and MAPK (45–47). We have indeed shown that inhibitors of both molecules significantly blocked the RhoC-dependent activation of MMP2 and MMP9. In this context, it should be noted that Ruth and colleagues have recently shown that RhoC promoted the invasion of human melanoma cells in a PI3K/Akt-dependent manner (48). Our results also indicate that Akt was significantly phosphorylated at Ser^{473} by RhoC, and that the phosphorylation of this serine residue has previously been found to be involved in the motility and invasiveness of tumor cells (45, 46, 49). The activation of Akt has also been shown to be clinically associated with aggressiveness and earlier recurrence of prostate cancer (50). Collectively, our results indicate that RhoC enhances the invasiveness and metastatic ability of tumor cells by

activating the Pyk2/FAK pathway followed by phosphorylation of Akt and MAPK, which in turn, activate MMP2 and MMP9. RhoC is considered to serve as an independent prognostic marker to predict patient outcome, and an intervention of the RhoC signal may be an effective therapeutic strategy for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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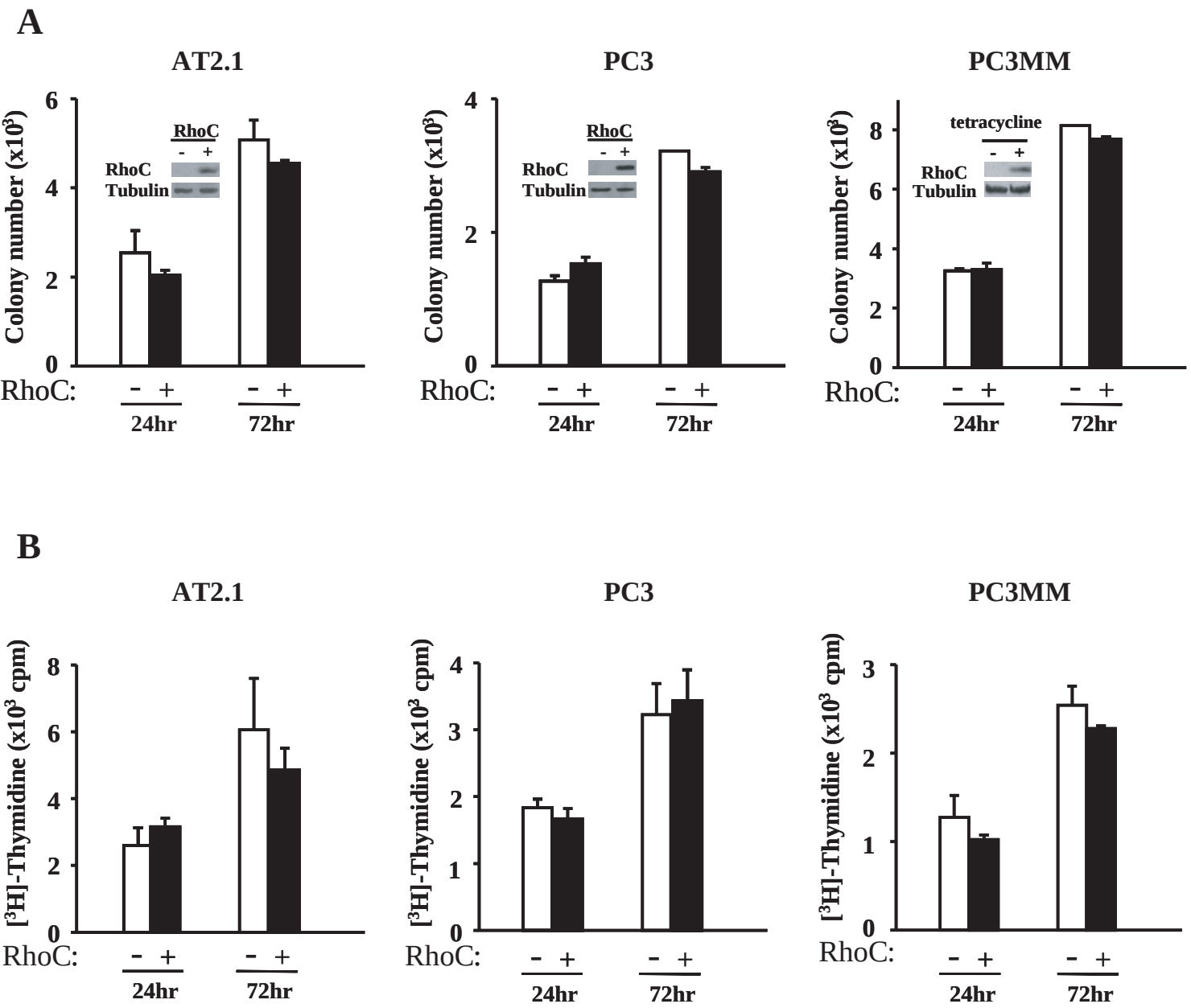
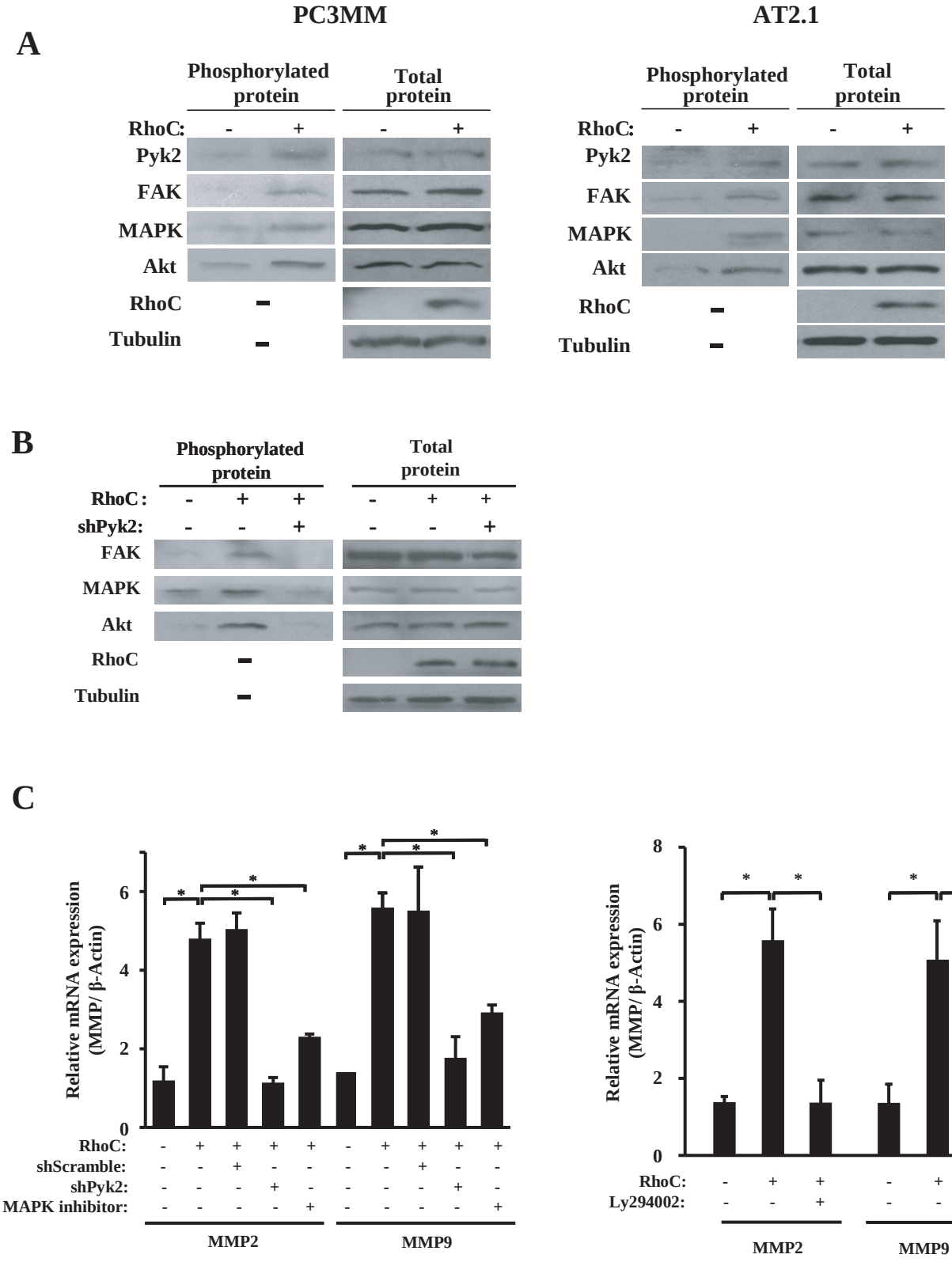


Supplementary Fig. S1 Iiizumi et al



Supplementary Fig. S2 Iiizumi et al